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(54) Title: PRODUCTION OF RECOMBINANT HUMAN ARYLSULFATASE A

(57) Abstract: The present invention relates to a cell capable of producing recombinant human ASA, said cell comprising the DNA fragment shown in SEQ ID NO 1. In particular, the invention relates to a cell comprising the 1578 basepair *EcoRI* - *XbaI* fragment of the DNA fragment shown in SEQ ID NO 2. One embodiment of the invention relates to an expression plasmid pAsaExp1 as shown in SEQ ID NO 2 for use in the expression of rhASA in cells. Further, the invention relates to a method for the preparation of recombinant human ASA, the method comprising a) introducing, into a suitable vector, a nucleic acid fragment comprising the DNA fragment shown in SEQ ID NO 1, b) transforming a cell with the vector obtained in step a), c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence, d) recovering the expression product from the culture. Optionally, the method may further comprise a fermentation step and/or purification step. The invention further relates to a rhASA produced by the method of the invention and to the use of the rhASA produced for the preparation of a medicament for the treatment of Metachromatic Leukodystrophy.

Production of recombinant human Arylsulfatase A

The present invention relates to a method for the production of the human Arylsulfatase A (ASA enzyme) useful for preventing or treating the development of symptoms related to
5 Metachromatic leukodystrophy (MLD) caused by a deficiency, in a subject, of said enzyme. In a further aspect the invention relates to a method of administering over cellular membranes, to a target cell, an effective amount of the human Arylsulfatase A (ASA) enzyme.

10 BACKGROUND OF THE INVENTION

Myelin metabolism and Metachromatic leukodystrophy

Metachromatic leukodystrophy (MLD) is caused by an autosomal recessive genetic defect
15 in the lysosomal enzyme Arylsulfatase A (ASA), resulting in a progressive breakdown of membranes of the myelin sheath (demyelination) and accumulation of galactosyl sulphatide (cerebroside sulfate) in the white matter of both central nervous system (CNS) and peripheral nervous system. In histologic preparations, galactosyl sulphatide forms spherical granular masses that stain metachromatically. Galactosyl sulphatide also
20 accumulates within the kidney, gallbladder, and certain other visceral organs and is excreted in excessive amounts in the urine.

Multiple sulfatase deficiency (MSD) is a rare form of MLD that also includes features of mucopolysaccharidosis (MPS). MSD is characterised by a decreased activity of all known
25 sulfatases. The clinical phenotype of MSD combines features of MLD with that of MPS as a result of the impaired lysosomal catabolism of sulphated glycolipids and glycosaminoglycans.

Galactosyl sulfatide is normally metabolised by the hydrolysis of 3-O-sulphate linkage to
30 form galactocerebroside through the combined action of the lysosomal enzyme arylsulfatase A (EC 3.1.6.8) (Austin et al. Biochem J. 1964, 93, 15C-17C) and a sphingolipid activator protein called saposin B. A profound deficiency of arylsulfatase A occurs in all tissues from patients with the late infantile, juvenile, and adult forms of MLD (see below). In the following, the arylsulfatase A protein will be termed "ASA" and the
35 saposin B will be termed "Sap-B". A profound deficiency of ASA occurs in all tissues from patients with MLD.

ASA has been purified from a variety of sources including human liver (Shapira E. Arch Biochemica Biophys. 1975, 170, 179-187, Draper RK et al. Arch Biochemica Biophys. 1976, 177, 525-538, James GT et al. Life sci. 1985, 37, 2365-2371), placenta (Farooqui AA. Arch Int Physiol Biochim. 1976, 84, 479-492, Gniot-Szulzycka J. Acta Biochim Pol. 1974, 21, 247-254), and urine (Stevens RL et al. J Biol Chem. 1975, 250, 2495-2501, Luijten JAFM et al. J Mol Med. 1978, 3, 213, Laidler PM et al. Biochim Biophys Acta. 1985, 827, 73-83). It is an acidic glucoprotein with a low isoelectric point. Above pH 6.5, the enzyme exists as a monomer with a molecular weight of approximately 100 kDa. ASA undergoes a pH-dependent polymerisation forming a dimer at pH 4.5. In human urine, the enzyme consists of two nonidentical subunits of 63 and 54 kDa (Laidler PM et al. Biochim Biophys Acta. 1985, 827, 73-83). ASA purified from human liver, placenta, and fibroblasts also consist of two subunits of slightly different sizes varying between 55 and 64 kDa (Draper RK et al. Arch Biochemica Biophys. 1976, 177, 525-538, Waheed A et al. Hoppe Seylers Z Physiol Chem. 1982, 363, 425-430, Fujii T et al. Biochim Biophys Acta. 1992, 1122, 93-98). As in the case of other lysosomal enzymes, ASA is synthesised on membrane-bound ribosomes as a glycosylated precursor. It then passes through the endoplasmic reticulum and Golgi, where its *N*-linked oligosaccharides are processed with the formation of phosphorylated and sulfated oligosaccharide of the complex type (Waheed A et al. Biochim Biophys Acta. 1985, 847, 53-61, Braulke T et al. Biochem Biophys Res Commun. 1987, 143, 178-185). In normal cultured fibroblasts, a precursor polypeptide of 62 kDa is produced, which translocates via mannose-6-phosphate receptor binding (Braulke T et al. J Biol Chem. 1990, 265, 6650-6655) to an acidic prelysosomal endosome (Kelly BM et al. Eur J Cell Biol. 1989, 48, 71-78).

The length (18 amino acids) of the human ASA signal peptide is based on the consensus sequence and a specific processing site for a signal sequence. Hence, from the deduced human ASA cDNA (EMBL GenBank accession numbers J04593 and X521151, see below) the cleavage of the signal peptide should be done in all cells after residue number 18 (Ala), resulting in the mature form of the human ASA. In the following, the mature form of the human ASA will be termed "mASA" and the mature recombinant human ASA will be termed "mrhASA".

Multiple forms of ASA have been demonstrated on electrophoresis and isoelectric focusing of enzyme preparations from human urine (Luijten JAFM et al. J Mol Med. 1978, 3, 213), leukocytes (Dubois et al. Biomedicine. 1975, 23, 116-119, Manowitz P et al. Biochem Med Metab Biol. 1988, 39, 117-120), platelets (Poretz et al. Biochem J. 1992, 287, 979-983), cultured fibroblasts (Waheed A et al. Hoppe Seylers Z Physiol Chem. 1982, 363, 425-430, Stevens RL et al. Biochim Biophys Acta. 1976, 445, 661-671, Farrell DF et al. Neurology. 1979, 29, 16-20) and liver (Stevens RL et al. Biochim Biophys Acta. 1976, 445, 661-671,

Farrell DF et al. Neurology. 1979, 29, 16-20, Sarafian TA et al. Biochem Med. 1985, 33, 372-380). Treatment with endoglycosidase H, sialidase, and alkaline phosphatase reduces the molecular size and complexity of the electrophoretic pattern, which suggests that much of the charge heterogeneity of ASA is due to variations in the carbohydrate content of the
5 enzyme.

The active site of ASA contains an essential histidine residue (Lee GD and Van Etten RL, Arch Biochem Biophys. 1975, 171, 424-434) and two or more arginine residues (James GT, Arch Biochem Biophys. 1979, 97, 57-62). Many anions are inhibitors of the enzyme at
10 concentrations in the millimolar range or lower.

A protein modification has been identified in two eukaryotic sulphatases (ASA and arylsulfatase B (ASB)) and for one from the green alga *Volvox carteri* (Schmidt B et al. Cell. 1995, 82, 271-278, Selmer T et al. Eur J Biochem. 1996, 238, 341-345). This
15 modification leads to the conversion of a cysteine residue, which is conserved among the known sulfatases, into a 2-amino-3-oxopropionic acid residue (Schmidt B et al. Cell. 1995, 82, 271-278). The novel amino acid derivative is also recognised as C α -formylglycin (FGly). In ASA and ASB derived from MSD cells, the Cys-69 residue is retained. Consequently, it is proposed that the conversion of the Cys-69 to FGly-69 is required for generating
20 catalytically active ASA and ASB, and that deficiency of this protein modification is the cause of MSD. Cys-69 is referred to the precursor ASA which has an 18 residue signal peptide. In the mASA the mentioned cysteine residue is Cys-51.

Further investigations have shown that a linear sequence of 16 residues surrounding the
25 Cys-51 in the mASA is sufficient to direct the conversion and that the protein modification occurs after or at a late stage of co-translational protein translocation into the endoplasmic reticulum when the polypeptide is not yet folded to its native structure (Dierks T et al. Proc Natl Acad Sci. 1997, 94, 11963-11968).

30 The human ASA gene structure has been described. In the following, this gene will be termed "ARSA". The ARSA gene is located near the end of the long arm of chromosome 22 (22q13.31-qter), it spans 3.2 kb (Kreysing et al. Eur J Biochem. 1990, 191, 627-631) and consists of eight exons specifying the 507 amino acid enzyme unit (Stein et al. J Biol Chem. 1989, 264, 1252-1259). Messenger RNAs of 2.1, 3.7, and 4.8 kb have been
35 detected in fibroblast cells, with the 2.1-kb message apparently responsible for the bulk of the active ASA generated by the cell (Kreysing et al. Eur J Biochem. 1990, 191, 627-631). The ARSA sequence has been deposited at the EMBL GenBank with the accession number X521150.

Differences between the published cDNA and the coding part of the ARSA were described by Kreysing et al. (Eur J Biochem. 1990, 191, 627-631). The cDNA sequence originally described by Stein et al. (J Biol Chem. 1989, 264, 1252-1259) and the cDNA sequence described by Kreysing et al. (Eur J Biochem. 1990, 191, 627-631) have been deposited at
5 the EMBL GenBank with the following accession numbers J04593 and X521151, respectively.

Several polymorphisms and more than 40 disease-related mutations have been identified in the ARSA gene (Gieselmann et al. Hum Mutat. 1994, 4, 233-242, Barth et al. Hum
10 Mutat. 1995, 6, 170-176, Draghia et al. Hum Mutat. 1997, 9, 234-242). The disease-related mutations in the ARSA gene can be categorised in two broad groups that correlate fairly well with the clinical phenotype of MLD. One group (I) produces no active enzyme, no immunoreactive protein, and expresses no ASA activity when introduced into cultured animal cell lines. The other group (A) generates small amounts of cross-reactive material
15 and low levels of functional enzyme in cultured cells. Individuals homozygous for an I-group mutation, or having two different mutations from this group, express late infantile MLD (see description of clinical manifestations of MLD below). Most individuals with one I-type and one A-type mutation develop the juvenile-onset form, whereas those with two A-type mutations generally manifest adult MLD. Some of the mutations have been found
20 relatively frequently, whereas others have been detected only in single families. It is possible to trace specific mutations through members of many families, however general carrier screening is not yet feasible.

In addition to the disease-related mutations described above several polymorphisms have
25 been identified in the ARSA gene. Extremely low ASA activity has been found in some clinically normal parents of MLD patients and also in the general population. This so-called pseudodeficiency ASA has been associated with a common polymorphism of the ARSA gene (Gieselmann et al. Dev Neurosci. 1991, 13, 222-227).

30 Sap-B, the sphingolipid activator, is a small heat-stable glycoprotein derived from a much larger precursor. This multiprotein precursor is referred to a prosaposin (pSAP) and its putative processing products, saposins A, B, C and D, appear to be part of a family of structurally related proteins involved in the catabolism of glycosphingolipids in lysosomes. Sap-B is necessary in the hydrolysis of sulphatide by ASA where it presents the solubilised
35 substrate to the enzyme. The prosaposin gen, is located on chromosome 10 and is not related to that for ASA. Mutations within the Sap-B region of the prosaposin gene have been identified in activator-deficient MLD patients. A small number of individuals with a deficiency in Sap-B have been reported and human total Sap deficiency has only been

reported in two patients in a single family. Most of these patients have had clinical signs typical of juvenile MLD, but ASA levels are within the normal range.

5 Clinical manifestations of MLD

The central nervous system consists of the brain and the spinal cord, and can be divided into white and grey matter. The white matter consists of nerve cells, and in MLD the damage occurs primary in the nerve cells. When the nerve cells are damaged, they can no longer conduct nerve impulses to muscles, skin and internal organs.

In cases of MLD, there is a defect in ASA activity affecting myelin metabolism. Lack of this enzyme in patients with MLD leads the degradation of myelin and to dysfunction of the nerve cells. A concomitant accumulation of special types of fat in the nerve cells is also observed in MLD.

Three forms of the disease can be distinguished according to the three forms of the age of onset: Late-infantile, juvenile and adult (after the age of 20 years).

The course of the disease varies in the different types. The type occurring in early childhood is the commonest, progresses most rapidly, and leads to pronounced handicapping and death.

In the infantile form of MLD there are several stages of the disease. The first stage is characterised by slack muscles (hypotonia) of the arms and legs. Walking deteriorates and the child needs support to walk. The picture is often complicated by disturbances of balance (ataxia) and weakened muscle reflexes. In the second stage, about 1-1½ years after the onset, the child can no longer stand, but it can still sit. The previous slack muscles become spastic. The disturbance of balance get worse, and pain in the arms and legs is common observed. The disease progresses to the third stage after additional 3-6 months where the child has increasing paralysis of all four limbs and can no longer sit. The child gradually needs help with everything, vision is impaired, and movements become difficult.

The juvenile type of MLD starts between the ages of five and ten years. The progression is similar to the infantile type, but slower. Emotional lability and impaired vision may be the first symptoms of the disease.

In the adult form of MLD the symptoms arise in the age after 20 years after normal development. The symptoms include cognitive and behavioural abnormalities.

Incidence of MLD

5

In Norway, about one child with MLD is born every year, i.e. a frequency of about 1:50.000. Similar results have been obtained in northern Sweden where the birth incidence rate for late infantile MLD in this population can be calculated to be about 1 per 40.000. Only one case of juvenile MLD was born in the mentioned region during the same
10 period (Gustavson et al. Acta Paediatr Scand. 1971, 60, 585-90. This demonstrates that the juvenile form of MLD is much more rare than the infantile form.

Existing diagnosis of MLD

15 In order to diagnose MLD, examination of spinal fluid, urine, various blood tests, and analysis of the ASA activity can be carried out. Deficiency of ASA activity in material from patients with MLD (e.g. peripheral leukocytes and cultured skin fibroblasts) can be investigated as described by Inoue et al. Experientia. 1986, 15, 33-35, Bradley et al. Anal
20 Biochem. 1988, 173, 33-38. Analysis of the urine from patients with MLD can indicate a defect at the level of myelin metabolism but this is a less reliable source for diagnostic assays because the urinary enzyme level is normally highly variable (ref 341-343 fra chapter 88). Excessive amounts of sulphatide excreted in the urine and metachromatic granules in the urinary sediment are observed. Furthermore, normal x-rays and computer
tomography (CT) of the head may be carried out.

25

Prenatal diagnosis appears to be possible by measuring ASA activity in cultured cells from amniotic fluid or chorionic villus cells. Cerebroside sulfate loading of such cells can also be used and is the method of choice if the pseudodeficiency gene is also present in the family.

30 Existing treatment of MLD

There are relatively few treatment options for MLD. Bone Marrow Transplantation (BMT) has been used in the treatment of more than 20 patients with MLD (Bayever E et al. Lancet 1985, 2, 471-473, Joss V et al. Exp Hematol 1982, 10, 52, Krivit W et al. Bone
35 Marrow Transplant 1992 10, 87-96) and it appears that BMT slows the progression of symptoms, but benefits of the treatment are not seen for several months. In most late infantile patients, symptoms are progressing rapidly by the time of diagnosis, and the risks of the procedure tend to outweigh the possible benefits. In instances in which the diagnosis can be made presymptomatically and a well-matched donor is available, BMT may

be a reasonable approach. Moreover, reported results suggest that BMT is efficacious only in MLD patients with high residual activity or when performed in presymptomatic stages in the late infantile form (Krivit et al. N Engl J Med 1990, 322, 28-32, Shapiro et al. J Inherit Metab Dis 1995, 18, 413-419) probably because of the rapid progression of the disease.

5

Cell culture models, suggests that cysteine protease inhibitor treatment (von Figura K et al. Am J Hum Genet 1986, 39, 371-382), thiosulfate treatment (Eto Y et al. Biochem Biophys Res Commun 1982, 106, 429-434), enzyme replacement (Porter MT Science 1971, 172 (1989), 1263-1265), and gene replacement therapies (Sangalli A et al. Hum Gene Ther 10 1998, 9, 2111-2119) could be effective. Several possible gene therapy approaches have been suggested.

In one of these approaches an implanted polymer-encapsulated xenogenic transduced cell line secreting the ASA enzyme is used. This approach has previously been used for the 15 treatment of other neurological disorders such as Amyotrophic Lateral Sclerosis and Parkinson disease (Aebischer et al. Nature Medicine 1996, 2, 696-699, Aebischer et al. Exp Neurol 1994, 126, 151-158). A cathetered device, containing around 106 genetically modified cells surrounded by a semipermeable membrane, is suggested to be implanted in the ventricular space, providing slow continuous release of ASA directly in cerebral spinal 20 fluid. For this gene transfer technique C2C12 mouse myoblast cells are used (Deglon et al. Hum Gene Ther 1996, 7, 2135-2146). The semipermeable membrane prevents immunologic rejection of the cells and interposes a physical barrier between cells and host. Moreover, the device and the cells may be retrieved in the event of side effect due to the ASA administration.

25

In another approach ARSA genes are directly delivered into the brain by the use of recombinant adenovirus (Ohashi et al. Acta Paediatr Jpn. 1996, 38, 193-201). It was shown that the recombinant adenovirus (Adex1SRLacZ) was able to transduce the oligodendrocytes very efficiently. Hence, it was concluded that the correction of ASA 30 deficiency by a recombinant adenovirus that potentially could be used to transfer the ARSA gene to the brain, and gene therapy for MLD based on gene transfer of the ARSA gene to mutant cells will be feasible, because the overexpression of ASA in cells does not lead to profound deficiency of other sulfatases or result in a new phenotype.

35 Animal model

Since no naturally occurring animal model of Metachromatic leukodystrophy (MLD) is available, Hess et al. (Proc Nat Acad Sci. 1996, 93, 14821-14826) generated ASA-deficient mice by targeted disruption of the gene in embryonic stem cells. Deficient animals stored

the sphingolipid cerebroside-3-sulfate in various neuronal and nonneuronal tissues.

Storage pattern was comparable with that in affected humans, but gross defect of white matter with progressive demyelination was not observed up to the age of 2 years.

Reduction of axonal cross-sectional area and astrogliosis was observed in 1-year-old mice;

- 5 activation of microglia started at 1 year and was generalised at 2 years. Purkinje cell dendrites showed altered morphology. In the acoustic ganglion numbers of neurons and myelinated fibers were severely decreased, which was accompanied by loss of brain stem auditory-evoked potentials. Neurologic examination demonstrated significant impairment of neuromotor coordination.

10

In addition to the results from Hess et al. mentioned above an ASA-deficient mice have been generated by homologous recombination (Gieselmann et al. J Inherit Metab Dis 1998, 21, 564-574). The murine ARSA gene and cDNA have been cloned and sequenced (Kreysing et al. Genomics. 1994, 15, 249-256). The ASA knockout mice are unable to

- 15 degrade sulphatide and store the lipid intralysosomally. The pattern of lipid storage in neuronal and non-neuronal tissues resembles that described for patients with MLD.

However, compared to humans the mice have a surprisingly mild phenotype, since they have a normal life span and do not develop widespread demyelination. ASA-deficient mice have been transplanted with bone marrow, which was transduced with a retroviral vector
20 expressing ASA. The majority of transplanted animals display sustained expression of ASA from the retroviral construct up to 5 months after transplantation. However, preliminary data suggest that this therapeutic approach does not reduce storage material (Gieselmann et al. J Inherit Metab Dis 1998, 21, 564-574).

- 25 As described above Sap-B deficiency causes a variant of MLD. With targeted disruption of the precursor protein gene, mice with total SAP deficiency have been generated (Oya et al. Acta Neuropathol. 1998, 96, 29-40). These mice developed progressive neurological symptoms around day 20 and could not survive beyond day 40.

30 Medical need in MLD

The lack of effective treatment for MLD is well recognised.

Children with MLD have great feeding problems. The doctor should be aware of the

- 35 nutritional status and consider adding extra calories to the food consumed in good time before skills are lost. As the feeding problems increase, it is important to start tube feeding, preferably a gastrostomy tube.

The prevention of MLD relies mainly on identifying carriers in known MLD families and providing genetic counselling with the possibility of prenatal diagnosis. Although usually reliable when several members of a family can be evaluated, carrier diagnosis based on half-normal levels of ASA is of questionable value for general screening.

5

In conclusion, there is a definite need for the provision of novel therapeutic methods aimed at this disease.

DISCLOSURE OF THE INVENTION

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In essence, the inventive concept of the present invention is based on the novel idea of substituting the reduced ASA enzymatic activity in the person having MLD simply by administering over cellular membranes, to a target cell, an effective amount of an ASA enzyme. This can thereby "assist" the enzyme that is in deficit.

15

Accordingly, in a first aspect, the present invention relates to a method for preventing or treating the development of symptoms related to Metachromatic leukodystrophy (MLD) caused by a deficiency, in a subject, of the human Arylsulfatase A (ASA) enzyme, the method comprising administering over a cellular membrane, to a target cell, an effective
20 amount of an ASA catalyst which is said enzyme or an enzymatically equivalent part or analogue thereof.

In a preferred embodiment, the present invention involves a treatment method in which a cellular barrier such as the blood-brain-barrier (BBB) is crossed whereby the material is
25 delivered to the target cells. Preferably, a vehicle such as a modified form of a protein, a peptide or fragments thereof and/or modified functional domains of toxins or fragments thereof will carry the material to the target cells.

The target cells for ASA therapy in CNS and the peripheral nervous system are the myelin
30 synthesising oligodendrocytes and the Schwann cells, respectively. Since the most severe symptoms caused by ASA deficiency are related to CNS, the system for delivering ASA to oligodendrocytes in the brain is the main objective of the enzyme replacement therapy. However, beneficial delivery of ASA to Schwann cells is also likely to occur. In addition, tissues (the kidney, gallbladder, and certain other visceral organs) with excretory function
35 are also affected by the enzyme defect, and should preferably also be targeted.

It is contemplated that effective enzyme replacement therapy of MLD patients with recombinant human ASA (rhASA) will require the uptake of an active enzyme into the target cells such as the myelin forming cells (oligodendrocytes) of the brain. To be able to

deliver rhASA to the brain a vehicle that can pass the blood-brain-barrier (BBB) is likely to be needed since rhASA is not likely to be able to traverse over the BBB by it self.

Enzymes can be delivered to oligodendrocytes in the brain directly via the cerebral spinal
5 fluid (CSF). Similar approaches have been reported for other neurological disorders such as amyotrophic lateral sclerosis and Parkinson disease (Aebischer et al. Nature Medicine 1996, 2, 696-699).

DEFINITIONS

10

By the term "catalyst" is herein meant either the relevant enzyme which is substituted as it is, or an enzymatically equivalent part or analogue thereof. One example of an enzymatically equivalent part of the enzyme could be a domain or sub-sequence of the enzyme which includes the necessary catalytic site to enable the domain or sub-sequence
15 to exert substantially the same enzymatic activity as the full-length enzyme.

An example of an enzymatically equivalent analogue of the enzyme could be a fusion protein which includes the catalytic site of the enzyme in a functional form, but it can also be a homologous variant of the enzyme derived from another species. Also, completely synthetic molecules which mimic the specific enzymatic activity of the relevant enzyme,
20 would also constitute "enzymatic equivalent analogues".

By the term "vehicle" is herein meant a peptide or a fragment thereof with a feature that allows it to mediate transversion of mrhASA or analogues over the BBB and/or cellular membranes.

25

By the term "hybrid molecule" is herein meant a fusion protein between the mASA catalyst and the peptide required for transport of the vehicle over the BBB and/or cellular membranes. In the said fusion protein the vehicle part can be attached to either the C-terminal or N-terminal end of the mASA catalyst.

30

By the term "mal folding" is herein meant a mediated process where an α -helix becomes a β -pleated sheet.

By the term "target cell" is herein meant a cell or group of cells (tissue) to which the
35 enzymes should be delivered.

DETAILED DISCLOSURE OF THE INVENTION

The present invention relates to a cell capable of producing recombinant human ASA, said
5 cell comprising the DNA fragment shown in SEQ ID NO 1. SEQ ID NO 1 encodes the
mature ASA and comprises the hASA signal sequence.

In a further aspect, the invention relates to a cell comprising the 1578 basepair *EcoRI* -
XbaI fragment of the DNA fragment shown in SEQ ID NO 2. SEQ ID NO:2 encodes the
10 mature ASA and comprises the hASA signal sequence and a Kozak site modulating the
initiation of translation and further comprises *EcoRI* and *XbaI* restriction sites which makes
it suitable for cloning expression vectors.

Further, the invention relates to a cell obtained by use of the expression plasmid pAsaExp1
15 having SEQ ID NO:2, in particular a cell obtained by transfection of a non-human
mammalian cell line, such as a chinese hamster ovary (CHO) cells. In particular, the
invention relates to a cell obtained by the culture of the human ASA production cell line
DSM ACC2550 which has been deposited at the DSMZ for the purposes of patent deposit
according to the Budapest Treaty on 6 June 2002.

20

In another aspect, the invention relates to a method for the preparation of recombinant
human ASA, the method comprising

a) introducing, into a suitable vector, a nucleic acid fragment comprising the DNA fragment
25 shown in SEQ ID NO 1;

b) transforming a cell with the vector obtained in step a);

c) culturing the transformed host cell under conditions facilitating expression of the nucleic
30 acid sequence;

d) recovering the expression product from the culture.

The method may further comprise a fermentation step and/or a purification step. In a
35 preferred embodiment of the method, the ASA is recombinant human ASA encoded by SEQ
ID NO 1. The invention also relates to a rhASA produced by the method of the invention
and to the use of the rhASA produced by the method of the invention for the preparation
of a medicament for the treatment of Metachromatic Leukodystrophy.

The invention further relates to an expression plasmid pAsaExp1 as shown in SEQ ID NO 2 for use in the expression of rhASA in cells.

In a further aspect, the present invention relates to a method for preventing or treating the development of symptoms related to Metachromatic leukodystrophy (MLD) caused by a deficiency, in a subject, of the human Arylsulfatase A (ASA) enzyme, the method comprising administering over a cellular membrane, to a target cell, an effective amount of an ASA catalyst which is said enzyme or an enzymatically equivalent part or analogue thereof. Preferably, the enzyme is rhASA produced by the method of the invention.

10

Preferably, the ASA catalyst is administered over a cellular membrane, to a target cell, by uptake of mASA into the target cell by taking advantage of a mannose-receptor-mediated uptake.

15 Mannose-6-phosphate tagged mASA is preferably made in a mammalian cell system (e.g. CHO, COS cells or BHK cells (Stein et al. J Biol Chem.1989, 264, 1252-1259) to secure correct mannose-6-phosphate tagging on the molecule, which ensures efficient receptor mediated uptake. Mannose-6-phosphate tagged mASA is secreted into the medium.

20 Examples 1- 6 provides an example of such production of mannose-6-phosphate tagged mASA.

Preferably, the mannose-receptor-mediated uptake of mASA into cells comprises one or several or all of the following steps (an outline):

25

A. Synthesis of mrhASA

Cloning of specific mrhASA cloning from human liver, placenta or spleen library

B. Transfection

30 2-10 µg mrhASA hybrid vector DNA is used for transfection by phosphate precipitate/ glycerol shock methodology, into mammalian cells (e.g. CHO, COS cells or BHK cells). Transfection might also be done with an electric shock methodology.

C. Expression of mrhASA

35 Conversion of the Cys-51 to FGly-51 and synthesis of the mannose-6-P is done during the expression in the mammalian cell system.

D. Purification of mrhASA

E. Test system for mannose-6-P receptor mediated uptake

The ability of produced mrhASA to be active in a mannose-6-P receptor mediated uptake is performed by incubating mrhASA with normal fibroblasts or fibroblasts from MLD patients.

- 5 Uptake into cells is assayed by increased ASA activity.

Example 3 shows an example of mannose-receptor-mediated uptake of mASA into cells.

- In the following different delivery techniques of mASA enzyme across the BBB and/or
10 cellular membranes are described. The ASA cDNA sequence deposit in the EMBL GenBank with the following accession numbers J04593 and X521151 encodes the ASA protein used in the present invention.

- 1) Peptides and proteins as vehicles for passage of mASA to the target cells by passage
15 over cell membranes and/or the BBB:

A number of studies in animals have shown that certain proteins and/or peptides may act as vehicles for passage of BBB. For instance proteins modified by the insulin fragment (Fukuta et al. Pharmacol Res 11: 1681-1688) or antibodies to the transferrin receptor (Friden et al. Proc Natl Acad Sci USA 88: 4771-4775) can pass the blood-brain barrier.

- 20 Also proteins modified by coupling to polyamines (Poduslo and Curran. J Neurochem 66: 1599-1606) have been reported to pass the blood-brain barrier.

2) Toxins as vehicles for passage mASA to the target cells by passage over cell membranes and/or the BBB:

- 25 Different bacteria, plants and animals produce toxins. Toxins have many different targets such as the gut (enterotoxins), nerves or synapses (neurotoxins). Toxins can traverse cell membranes via receptor mediated processes and one embodiment of the present invention is to use toxins as vehicles to passage rhASA to the target cells over cellular membranes and/or the BBB. The preferred target cells are cells in the CNS and/or the peripheral
30 nervous system.

A further embodiment of the present invention is that only the peptide pertaining to the translocation over cellular membranes and/or the BBB of the toxin is used.

- 35 One example of a toxin used as a vehicle is a bacterial toxin such as Diphtheria Toxin (DT), from the *Corynebacterium Diphtheriae*. Bacterial toxins exhibit a wide range of toxicities and they fall into groups by structure and function. The toxin binds to a target cell and enters the cell via a receptor, and is reduced to separate fragments. The processed toxin

can be divided into the following 3 domains: The catalytic domain (C), the receptor domain (R), and the translocation domain (T).

The catalytic fragment and the receptor fragment of the toxin or fragments thereof are replaced by the mASA. This fusion protein can traverse cellular membranes and/or the BBB and thereby deliver the mASA to the target cells. One example of the engineering of a hybrid molecule by recombinant technology comprises one or several or all of the following steps (an outline):

10 A. Synthesis of mrhASA

Cloning of specific mrhASA cDNA from human liver, placenta or spleen library.

B. Synthesis of DT cDNA

1. Cloning of Diphtheria Toxin cDNA.
- 15 2. Removal of the cDNA coding for the catalytic domain and the receptor domain or fragments thereof. The remaining fragment of DT is in the following termed "DT(T).

C. Construction of the DT(T)-mrhASA hybrid

1. Ligation of mrhASA cDNA with the DT(T) fragment.

20

D. Expression

1. Ligate the DT(T)-mrhASA construct in a vector for efficient expression in a mammalian cell system e.g. COS, BHK or CHO.
2. Conversion of the Cys-51 to FGly-51 and the synthesis of mannose-6-P tag on the
- 25 hybrid protein occur during the expression in the mammalian cell system.

Other examples of bacterial toxins used as vehicles are Clostridium Botulinum, Pseudomonas Exotoxin A produced by *Pseudomonas aeruginosa*, Cholera Toxin produced by *Vibrio cholerae*, and Pertussis Toxin produced by *Bordetella pertussis*.

30

Further examples of toxins used as vehicles are plant toxins selected from the list of the following plant toxins: cholinesterase inhibitors, protease inhibitors, amylase inhibitors, tannins, cyanogenic glycosides, goitrogens, lectin proteins, and lathrogens, pyrroizidine alkaloids.

35

Yet further examples of toxins used as vehicle are toxins from shellfish (saxitoxin) and snakes (alpha-bungarotoxin). The skilled person may add further examples to list in light of the details and surprising characteristics of the invention. It is evident to the person of

ordinary skill in the art that a fusion protein comprising any of the disclosed toxins or a part thereof could be prepared in a similar manner as outlined with respect to DT above.

3) Proteins and/or peptides isolated from bacteria or viruses as vehicles for passage mASA to the target cells by passage over cell membranes and/or the BBB:

5

The transacting element and/or transacting protein from bacteria or viruses can be used together with mASA to cross the BBB and/or cellular membranes.

In one aspect, a protein or peptide derivable from a virus may be used. However, as virus
10 in its nature has the cell nucleus as target organ for the infection, it is contemplated that a modification or specific fragment not having this effect may be a preferred embodiment. The delivery of an enzyme to an enzyme deficient cell related to MLD by use of an protein virus is a further surprising aspect of the invention.

15 As virus in general is an inhomogeneous group with respect to affinity for different cells, it is within the spirit of the present invention to select the concrete virus in accordance with the desired target cell to which the enzyme is to be delivered. One method of the invention relates to the use of a protein or peptide derivable from a virus or belonging to any of the families including Parvoviridae, Papovaviridae, Adenoviridae, Herpesviridae, Poxviridae,
20 Picornaviridae, Reoviridae, Togaviridae, Arenaviridae, Coronaviridae, Retroviridae, Bunyaviridae, Orthomyxoviridae, Paramyxoviridae, and Rhabdoviridae, the preferred viruses being selected from Measles virus, Papova virus, and JC virus.

In a further aspect, the protein or peptide is derivable from a bacterium such as a
25 bacterium selected from the group comprising *Ns. meningitidis*, *S. pneumoniae*, *Hemophilus influenzae*, *Staphylococcus* species, *Proteus* species, *Pseudomonas* species, *E. coli*, *Listeria monocytogenes*, *M. tuberculosis*, *Neurospora*, and *Spirochetes Borrelia burgdorferi* from *Iodex ricinus*.

30 4) Mannose-receptor mediated uptake of ASA into cells:

Several human cells (monocytes, fibroblasts, lymphocytes) have shown to be able to cross the BBB by it-self (Hickey WF, Kimura H. Science. 1988, 239, 290-292, Hickey WF et al. J Neurosci Res. 1991, 28, 254-260). These cells can be "loaded" with mature ASA (mASA) and can act as a vehicle for transport of mASA to the brain. Preferably, lymphocytes are
35 used as vehicles, because they have a long half-life (2-3 months). Uptake of mASA into lymphocytes will take advantage of a mannose-receptor-mediated uptake.

Mannose-6-phosphate tagged mASA is made in a mammalian cell system (e.g. CHO, COS cells or BHK cells (Stein et al. J Biol Chem. 1989, 264, 1252-1259) to secure correct

mannose-6-phosphate tagging on the molecule, which ensures efficient receptor mediated uptake. Mannose-6-phosphate tagged mASA is secreted into the medium and purification of rhASA is facilitated by the use of ammonium salts (NH_4Cl) in the fermentation step.

- 5 mASA has three putative N-glycosylation sites i.e. Asn158, Asn184, and Asn 350, which can form the mannose-6-P tag. Asn158, Asn184, and Asn350 are referred to in the precursor ASA which has an 18 residue signal peptide. In the mature ASA the mentioned asparagine residues are Asn140, Asn166, and Asn332, respectively. Only two of the N-glycosylation sites (Asn140 and Asn332) undergo phosphorylation and can acquire the
10 correct mannose-6-P tag and the mannose-6-P synthesis at these two sites via two distinct enzymatic steps is shown in Fig. 3.

- The disease which is the target for the method of the invention is MLD, and therefore the catalyst is ASA or an enzymatically equivalent part or analogue thereof. It is most
15 preferred that the catalyst is a human recombinant form of the ASA enzyme or of the enzymatically equivalent part or analogue thereof, since recombinant production will allow large-scale production which, with the present means available, does not seem feasible if the enzyme would have to be purified from a native source.

- 20 Furthermore, use of such catalysts, as described herein, for the preparation of a pharmaceutical composition for treatment of the above-discussed diseases is also part of the invention.

- Thus, the present invention represents an important advance in the treatment of genetic
25 and/or acquired metabolic brain disorders of MLD without the problems associated with prior treatment methods, including the gene therapy and bone marrow transplantation.

- The present invention relates to the new and surprising concept of use as a carrier of a peptide or protein from a structure capable of crossing a biological barrier, such as a
30 cellular barrier, including the blood-brain-barrier or a specific membrane of a cell. The object of the carrier function is to deliver an enzyme to a target cell. The target cell is generally a cell wherein the enzyme activity is insufficient either due to a decreased activity of the enzyme or to a situation where an increased activity is desired.

- 35 In one embodiment, the invention relates to a method for increasing the content of an enzyme in a cell comprising delivery of the enzyme to the tissue relevant for the cell and/or to the cell by use of a protein or peptide capable of crossing a cellular barrier, the protein or peptide being derivable from the group of toxins, bacteria, and from fragments and modifications thereof.

The disease MLD has been explained in detail above and the present invention is of particular importance in connection with alleviating the progression of symptoms caused by the enzyme defect of ASA. Accordingly, in a preferred embodiment, the invention
5 relates to a method for preventing or treating the development of symptoms related to Metachromatic leukodystrophy (MLD) comprising the administration of the enzyme arylsulfatase A (ASA) to the tissue relevant for the cells and/or to the cells by use of a protein peptide capable of crossing a cellular barrier or by use of a human cell as a vehicle for delivering of the enzyme to the tissue relevant for the cells and/or to the cells.

10

As explained in the present text, specific modification of a protein vehicle by deletion of parts of the peptide sequence can be obtained whereas the part or parts of the protein relevant for the protein transduction is retained. In addition to deletion, substitution of specific amino acids may also be performed in order to modify the protein to a perform the
15 carrier function without undesired side effects.

Similar to the use of a protein or peptide the present invention also relates to the use of peptide or proteins generally referred to as toxins as described above. Such a toxin may be a toxin selected from plant toxins, bacterial toxins and from toxins from animals. In
20 accordance with the disclosure for the proteins the toxins may be modified in order to increase the desired properties of the peptide.

Affection of the nervous system, especially of the central nervous system, very often plays a significant role in the broad range of diseases caused by enzyme deficiency. Accordingly,
25 in a preferred embodiment, the carrier protein or peptide is one which is capable of crossing the blood-brain-barrier.

This is possible by use of proteins or peptide which due to their nature bears a structure relevant for such transduction. In an important method according to the invention, the
30 cellular barrier includes the blood-brain-barrier (BBB) and the target is a tissue and/or cell of the central nervous system. An important target cell is a cell of the brain such as an oligodendrocyte. A further relevant cell is CG4 cell.

Important target cells are also cells or tissue relating to the peripheral nervous system
35 including a Schwann cell.

The overall idea of the present invention is to prepare a construct comprising the enzyme in question and the delivering protein/peptide. Accordingly, in one aspect the invention

relates to a method wherein the enzyme forms a hybrid with the protein or peptide capable of crossing the cellular barrier.

As explained in further details in the text of the present specification the hybrid is
5 preferably produced recombinantly. However, the construct may be produced by techniques of protein synthesis generally known in the art including solid phase synthesis. The complete hybrid or part of the hybrid may accordingly be produced synthetically or a part or the hybrid construct may be produced by use of a genuine protein or peptide. The enzyme part and delivery part may be linked by different techniques known in the art.

10

Preferably, the ASA is made recombinantly. In a further embodiment, the ASA is human ASA and still more preferred mature human ASA (mASA) or a fragment thereof. The fragment may be modified, however the active sites of the enzyme should be preserved. One example of a modification pertains to the conversion of the Cys-51 to FGly in the
15 mature human ASA.

In a still further aspect of the invention, cells are used as a vehicle for delivering an enzyme, preferably ASA, to the target cell. The preferred human cell is selected from human monocytes, human fibroblasts, and human lymphocytes. As explained above, it is
20 preferred for target cells of the central nervous system that the cell for delivering the enzyme is capable of crossing the BBB for delivering the ASA to the tissue and/or cells of the central nervous system.

In order to facilitate the delivery of the enzyme to the target cell, the ASA may be
25 transferred to a target cell by means of a mannose-receptor-mediated uptake. Such uptake may be further increased when the ASA is a mannose-6-P tagged ASA, preferably made by expressing ASA in a mammalian cell system. A preferred mammalian cell system is selected from the group consisting of CHO cells, COS cells, and BHK cells.

30 In general, the target cell is a cell wherein the activity, such as ASA, is insufficient for the optimal function of the cell. Insufficient activity of ASA may be measured by one or more of the parameters selected from increase in urinary sulfatide excretion, analysis of ASA activity in material from the patient such as in leukocytes and/or in skin fibroblasts, decreased nerve conduction velocity in the patient, CT scanning and/or Magnetic
35 Resonance Imaging of the patient, presence of clinical symptoms or increase in rate of development of clinical symptoms of MLD.

A significant feature of insufficient ASA activity is a cell wherein an accumulation of galactosyl sulphatide is present. Naturally, such cell is a target cell according to the

present invention. The target cell may also be cells of the kidney, gallbladder, liver or other visceral organs which very often are affected in addition to the cells of the nervous system. In longterm treatments, it may be important to preserve the optimal function of the above-mentioned organs. In addition, target cells for delivering the enzyme also
5 includes one or more cell types selected from the group consisting of human monocytes, human fibroblasts, human lymphocytes and human macrophages.

An increased activity of the ASA may be used as a parameter for a treatment schedule and may be measured by one or more of the parameters selected from decrease in urinary
10 sulfatide excretion, analysis of ASA activity in material from the patient such as in leukocytes and/or in skin fibroblasts, increased nerve conduction velocity in the patient, CT scanning, Magnetic Resonance Imaging, decrease in clinical symptoms/decrease in rate of development of clinical symptoms.

15 It is a very important aspect of the invention to perform the treatment of a possible enzyme defect prenatally. In a further aspect of the invention, the cellular membrane is the fetal-maternal barrier (placenta). It is also within the scope of the invention to deliver the enzyme-protein-construct directly to the fetus prenatally.

20 In a still further embodiment, the invention relates to an antibody raised against any of the constructs formed by any of the enzymes and any of the proteins and/or peptide mentioned herein. Such antibody may be used for the targeting of the construct, e.g. for inactivation of the construction including increasing the elimination of the construct from the subject. The antibody may be a polyclonal antibody or a monoclonal antibody and may
25 be produced by techniques known in the art.

Any enzyme construct disclosed above as well as the antibody thereto may be used for the preparation of a medicament for a treatment in accordance with the method disclosed in the present context. Accordingly, the present invention also relates to a pharmaceutical
30 medicament comprising an enzyme linked to a carrier system such as a protein or peptide or to a cell system as disclosed in detail above.

Accordingly, in one aspect, the present invention relates to a construct as well as to the use of a construct comprising an enzyme and/or a protein or peptide capable of crossing a
35 cellular barrier or a human cell as a vehicle for delivering of the enzyme to the tissue relevant for the cells and/or to the cells as disclosed in any of the methods mentioned and specified in the claims for the preparation of a medicament.

Also the antibody and use thereof for the preparation of a medicament is within the scope of the invention.

LEGEND TO FIGURES

5

Fig. 1 represents a schematically presentation of the hybrid molecule DT(T)-mASA. Two domains (the receptor domain (R) and the catalytic domain (C)) of the bacterial toxin Diphtheria Toxin (DT) are exchanged with the mASA fragment.

- 10 Fig. 2 represents a flow chart that illustrates the Mannose-6-P tagging of the mature human ASA (mASA 19-507). ASA has three putative N-glycosylation sites (Asn158, Asn184, and Asn350, which can form the mannose-6-P tag). Asn158, Asn184, and Asn350 are referred to the precursor ASA which has an 18 residue signal peptide. In the mASA the mentioned asparagine residues are Asn140, Asn166, and Asn332, respectively.

15

Fig. 3 Restriction map PUCspASA. Note that there are two SAcII sites on the figure. All other sites are unique. Bla is beta lactamase. spASA is ASA coding region, see Fig 6.

- Fig. 4 Restriction map of pAsaExp1. "pCMV" designates the Human cytomegalovirus immediate-early promoter/enhancer region; "Intron" is a Chimeric Intron; "rhASA" is the coding region of rhArylsulfatase A; "SV40-term" is the SV40 late polyadenylation signal; "f1" is the phage f1 region; "SV40 Eprom" is the SV40 early promoter/enhancer and origin; "DHFR" is mouse dihydrofolate reductase coding region; "pA" is a synthetic polyadenylation signal; Amp^R is beta-lactamase (Amp^R) coding region.

25

- Fig 5. SDS/PAGE analysis of anion exchange chromatography for rhASA. Gels are 4-12% BisTris w MES/SDS reducing. The arrow indicates to position of rhASA on the gel. The contents in each lane are as follows: lane 1: Markers; lane 2: Start mixture; lane 3: Flow Thru; lane 4: fraction 23; lane 5: fraction 24; lane 6: fraction 25; lane 7: fraction 26; lane 8: fraction 27; lane 9: fraction 28; lane 10: fraction 29; lane 11: fraction 30; lane 12: fraction 32; lane 13: fraction 37; lane 14: Markers; lane 15: fraction 41; lane 16: fraction 43; lane 17: fraction 46; lane 18: fraction 48; lane 19: fraction 52; lane 20: fraction 55; lane 21: fraction 58; lane 22: fraction 74; lane 23: fraction 78; lane 24: fraction 82. See also table 1, example 2.

- 35 Fig 6. MLD fibroblasts GM00197 loaded with ConA-purified rhASA for 3 days in serum-free medium.

Fig 7. MLD fibroblasts GM00197 loaded with affinity-purified rhASA for 3 days in serum-free medium.

Fig 8. MLD fibroblasts GM00243 loaded with affinity-purified rhASA for 3 days in serum-free medium with the addition of 5 mM Mannose-6-Phosphate (M-6-P) or Glucose-6-Phosphate (G-6-P).

Fig 9. MLD fibroblasts GM00243 loaded with affinity-purified rhASA for 3 days in serum-free medium. Loaded cells were stained with a green lysosomal marker, LAMP-1-FITC, (A and B) and a red color for the rhASA enzyme. This was done using a monoclonal antibody specific for rhASA and a secondary RAM-TRITC antibody (C and D). Control stainings were negative (not shown). When the red and green staining was overlaid, a yellow/orange color showed co-localization (E and F).

Fig 10. MLD fibroblasts GM00243 loaded with affinity-purified rhASA (0 or 100 mU/ml) for 1-8 hours in complete or serum-free medium.

Fig 11. MLD fibroblasts GM00243 loaded with affinity-purified rhASA (0 or 100 mU/ml) for 15-69 hours in complete or serum-free medium.

Fig 12. MLD fibroblasts GM00243 loaded with affinity-purified rhASA (0 or 100 mU/ml) for 24 hours in complete medium. After this, cells were washed and complete medium without rhASA was added. Cells were trypsinated when confluent at time-points 0 hours (and subcultivated 1:2), 48 hours (and subcultivated 1:5) and 120 hours (end of experiment). Intracellular ASA activity and total protein content were measured at all time-points.

Fig 13. Growth curve for CHO-ASA cells cultivated in a shake flask (160 mL). Cells were inoculated (2.5×10^5 cells/ml) at time-point zero and then followed for 100 hours when they were harvested (1.35×10^6 cells/ml). The cell viability was 75-90 % during the whole cultivation. Total number of cells (Nt), number of viable cells (Nv).

Fig 14. ASA production (mU/mL) in two shake flask cultures (120-150 ml), one cultivated with 20 nM MTX and the other without any MTX for 5 weeks.

Fig 15. Comparison of ASA-CHO cell growth for batch 1 & 2. Triangles describes values from batch #1, squares describes values from batch #2.

Fig 16. Comparison of ASA production for batch 1 & 2. Triangles describes values from batch #1, squares describes values from batch #2.

EXAMPLES

Example 1: Cloning, construction of expression vector, and expression of ASA in Chinese Hamster Ovary (CHO) cells

5

Materials and methods

Materials used for cell culture

Media:

10	<u>Material</u>	<u>Company</u>	<u>Catalogue #</u>
	Penicillin/Streptomycin	BioWhittaker	17-602E
	L-Glutamine, 200 mM	BioWhittaker	17-605E
	HT Supplement (100X)	Gibco BRL	11067-030
	Ex-Cell 302 Serum-Free Media	JRH BioSciences	14312-78P

15

Media for DG44.42 Cells:

	Ex-Cell 302 Serum-Free Medium	500 ml
	Penicillin/Streptomycin	5 ml
	L-Glutamine (4 mM final concentration)	10 ml
20	100X HT Supplement	5 ml

Media for ASA Masterwell Cells:

	Ex-Cell 302 Serum-Free Medium	500 ml
	Penicillin/Streptomycin	5 ml
25	L-Glutamine (4 mM final concentration)	10 ml
	20nM Methotrexate	0.5 ml (20 μ M MTX*)

*Diluted from 20 mM Stock.

30 Preparation of 20 mM Methotrexate Stock:

Materials:

Methotrexate, U.S.P.: ICN Catalogue # 102299, 100 mg

WFI (Water for Injection)

1.0 N NaOH to adjust pH.

35 0.2 μ m filter unit of appropriate size.ASA enzyme assay

Materials:

50 mM p-Nitrocatechol Sulphate:

Dissolve 0.156 g p-Nitrocatechol Sulphate (Sigma, Catalogue # N-7251) in 10 ml double distilled (dd) H₂O. Store at 4°C in foil.

3 M Sodium Acetate pH 5.0:

- 5 Dissolve 24.69 g sodium acetate in 100ml dd H₂O, adjusted to pH 5 and store at room temperature.

4X Assay Buffer (should be made fresh on day of use):

- 10 Mix 5.0 ml 3 M sodium acetate pH 5.0, 6.0 ml 50 mM p-Nitrocatechol Sulphate and 4.0 ml dd H₂O.

1 M NaOH:

4g NaOH + 100 ml dd H₂O, stored at room temperature.

15 Procedure:

For screening purposes the assays were done in flat-bottomed Elisa plates. 25 µl of the 4X assay buffer was added to 75 µl of sample or an appropriate dilution of it. The plates were incubated overnight at 4°C, stopped with 200 µl of 1 M NaOH and the absorbance recorded at 515 nm on a plate reader.

20

For determination of specific activity of the DEAE-purified samples, the assays were set up in tubes with all the volumes doubled. Incubations were at 37°C for periods ranging from 5-20 minutes using 10-1000 ng of enzyme. The samples were read on a spectrophotometer using a cuvette of 1 cm path length. Specific activity is defined as 25 µmoles of p-Nitrocatechol Sulphate hydrolysed per minute per mg protein at 37°C, pH 5.0.

Gel electrophoresis

Gel electrophoresis was done using the Novex system with Nupage Bis/Tris gels run at 200 volts for 1.2 hours. Staining was done with Coomassie brilliant blue R-250 according to 30 the manufacturer's specifications. Total protein was measured by the Bradford method using the BioRad reagent (Catalogue # 500-006) and bovine serum albumin as standard.

PCR and Cloning of mature polypeptide

- 35 Two cDNA libraries served as the source for cloning the cDNA encoding the mature polypeptide of human ASA by PCR amplification. One was a Hep G2 cDNA library made from mRNA isolated from the Hep G2 cell line (ATCC # HB-8065) using the Superscript Plasmid System manufactured by Gibco BRL, Catalogue # 18248-013. The other library

was ICO 289 cDNA library made from mRNA derived from the human skin fibroblastic cell line EB91-289 (Jack Oram, University of Washington).

Two hundred ng of DNA from the libraries were amplified with Advantage cDNA
5 polymerase mix (Clontech Catalogue # 8417-1) with 0.2 mM dNTP and 0.4 μ M each of
ICO720 (5' ACGTTAGAATTCGTTGCACGTCCGCCCAACA 3' (SEQ ID NO. 3)) and ICO721
(5' GTCGACTCTAGACCAGTGAGGAGCCATCACAT 3' (SEQ ID NO. 4)) in 50 μ l reaction
volumes, resulting in a 1542 bp product. Two cycle PCR was used with an initial heat
denaturation step at 94°C for 100 seconds followed by 30 cycles of 94°C for 20 seconds
10 and 72°C for 3 minutes. A final extension of 10 minutes at 72°C was used at the end to
ensure that the extension products were filled out. The PCR product was cloned into pUC19
linearized with *Eco*R I and *Xba* I after purification (using GENECLEAN III, from BIO 101
Catalogue # 1001-600) followed by digestion with the same two enzymes. The resulting
clones were named pUCasa-1-6.

15

Sequencing

The six plasmid clones from the ligation described above named pUCasa-1-6 were
sequenced with the Big dye terminator cycle sequencing kit from PE/ABI (Catalogue #
4303152).

20

The completed sequencing effort revealed that clone #2 had the correct sequence.
Preliminary sequencing results unambiguously showed that the 5' segment of clone #1 and
the 3' end of clone #4 were also correct. Employing a *Sac* II site at position 729 in the ASA
sequence, a hybrid clone named pUCasa1-4 was constructed. A 670 bp *Eco*R I-*Sac* II was
25 isolated from pUCasa-1 and a 835 bp *Sac* II-*Xba* I fragment from pUCasa-4. These were
assembled into pUC19 linearized with *Eco*R I and *Xba* I in a three-part ligation to generate
plasmid pUCasa1-4. This plasmid served as the source of cDNA of the mature ASA
polypeptide for all subsequent constructions. The ASA insert in pUCasa1-4 was confirmed
by DNA sequencing.

30

Construction of full-length cDNA

The full-length cDNA including the endogenous signal peptide sequence was assembled
into pUC19. Plasmid pUCasa1-4 was digested with *Btr* I and *Xba* I and the 1512 bp
fragment encoding the mature ASA polypeptide was isolated. This was combined with
35 oligonucleotides ICO746

(5'AATTCGCCACCATGGGGGCACCGCGGTCCCTCCTCGGCCCTGGCTGCTGGCCTGGCCG
TTGCAC 3' (SEQ ID NO. 5)) and ICO747 (5' GTGCAACGGCCAGGCCA GCAGCCAGGG
CCAGGAGGAGGGACCGCGGTGCCCCCATGGTGGCG 3' (SEQ ID NO. 6)) and ligated into
pUC19 linearized with *Eco*R I and *Xba* I to generate pUCspASA. The sequence of the

adaptor comprising of oligonucleotides ICO746 and ICO747, encompassing the signal peptide and the Kozak site (Kozak M. Structural features in eukaryotic mRNAs that modulate the initiation of translation (*J Biol Chem.* 266[30], 19867-19870. 1991) was confirmed by DNA sequencing.

5

The full-length ASA clone named pUCspASA constructed from pUCasa1-4 and synthetic adaptors has been sequence confirmed and matches the published sequence (Stein C, Gieselmann V, Kreysing J, Schmidt B, Pohlmann R, Waheed A, Meyer HE, O'Brien JS, von Figura K. Cloning and expression of human arylsulfatase A. *J.Biol.Chem.* 264, 1252-1259. 10 1989). A map of pUCspASA is shown in Fig. 3 and the nucleotide sequence of the ASA coding region of the plasmid is shown in SEQ ID NO. 1.

Construction of the expression plasmid

The vector used for expression is derived from the expression vector pCI-neo obtained 15 from Promega, Catalogue # E1841. The neomycin phosphotransferase (neo) gene was replaced by the mouse DHFR gene and the region upstream of the neo gene up to the *Dra* III site with the corresponding region from pcDNA3.1(+) from Invitrogen, Catalogue # V790-20. The resulting vector is called pCI-DHFR. The final expression plasmid pAsaExp1 was obtained by cloning the *EcoR* I-*Xba* I fragment from pUCspASA encoding ASA into pCI- 20 DHFR between the same sites. A map of pAsaExp1 is shown in Fig. 4 and the complete nucleotide sequence of the plasmid is shown in SEQ ID NO. 2.

Transfection and culture conditions

8x10⁶ CHO DG44.42 cells were resuspended in 800 µl Hepes Buffered Electroporation 25 Media with 100 µg pAsaExp1 DNA (linearized with *Dra* III) +100 µg salmon sperm DNA in a 0.4 cm electroporation cuvette. The cells were electroporated using a Bio-Rad Gene Pulser II set at 300 volts with 950 uFd capacitance. The contents of the cuvette were then transferred to a T-75 flask containing 20 ml of JRH Ex-Cell 302 Serum-Free Medium + HT + 4mM L-glutamine + 100 units/ml penicillin + 100ug/ml streptomycin and then placed in 30 a 37°C, 5% CO₂ incubator. 24 hours later the cells were counted, centrifuged and transferred to two T-flasks, one with 20 nM and the other with 50nM methotrexate (MTX) replacing the HT in the growth media described above. A media sample was saved and run in an ASA activity assay. The cells were subcultured in this manner for 18 days with regular shifts of medium. On day 18 the cells were seeded at 200 cells/well in the 35 respective methotrexate concentrations in a 96 well plate. Three weeks later an ASA activity assay was done on media from wells containing 1 to 3 clones. The masterwells with the highest ASA activity were then expanded and run in a 6-day ASA activity assay. In a 6-day assay cells are seeded at 1x10⁵ cells/ml in 5 ml media with methotrexate in a T-12.5 flask. 6 days later the cells are counted. The cells are then centrifuged and the

supernatant is analyzed in the ASA activity assay described below. Results were normalized for cell number. The 14 best masterwells were then amplified - see example 2.

Conclusion

5 Full length cDNA encoding recombinant human arylsulfatase A (rhASA) was cloned into pUC19. The resulting plasmid pUCspASA served as the source of rhASA cDNA. This cDNA was transferred into a mammalian expression vector pAsaExp1 which in addition to rhASA also carries the mouse dihydrofolate reductase (DHFR) cDNA as the selectable marker. DHFR can be used for further amplification of the plasmid with methotrexate.

10

The DNA sequence of pAsaExp1 was verified by sequencing and by transfection experiments that shows that pAsaExp1 encodes a functional human arylsulfatase A.

Example 2: Selection of producer cell and preliminary characterization of recombinant

15 human ASA.

Materials and methods

For media composition, ASA enzyme assay and gel electrophoresis details, see materials and methods in example 1.

20

To obtain the final clonal cell line expressing rhASA, the expression plasmid pAsaExp1 (Fig 4, SEQ ID NO. 2) was transfected into the host cell line DG44.42 (obtained from DG44 by limiting dilution). High ASA producing masterwells, as determined by ASA activity, were selected - see example 1.

25

To further increase the yield, the cells from high ASA producing masterwells were subjected to amplification with methotrexate (MTX) as described by Gasser et al. Proc. Natl. Acad. Sci. U.S.A 79, 6522-6526. 1982. After amplification, the best producers were cloned twice by limiting dilution.

30

Amplification

Briefly, ten masterwells were amplified starting from 20 nM MTX and two starting at 50 nM MTX. Amplification was done by seeding cells at 1×10^5 cells/ml in T-12.5 or T-25 Falcon flasks, in 5 or 10ml of media respectively. The cells were then split every 4-6 days and
35 analyzed in a 6-day assay (see example 1 for assay details). The cells were then amplified to the next level (50, 100, 200, 500 and 1000 nM MTX) on the next split as well as the cells being maintained at previous MTX levels. Eventually, the MTX levels reached 500 and 1000 nM MTX. The cells did not grow as well at these levels or produce more than at 200 nM MTX.

Five masterwells were followed to the end. 4B3 and 4G2 started at 50 nM MTX did not amplify. 1G2, 2B4 and 3F6 were 9%, 0% and 20% higher regarding ASA production at 200 nM than at 20 nM MTX.

5

1st Round Limiting Dilution Cloning

Limiting dilution cloning was begun during the amplification process at 200 nM and 500 nM MTX. Three masterwells at 200 nM MTX (1G2, 3F6 and 4B3) were seeded at 50, 100 and 200 cells/well and two masterwells at 500 nM MTX (4B3 and 4G2) were seeded at 200
10 cells/well in 100 µl media in a flat-bottom 96- well plate. They were fed 100 µl fresh media every 5-7 days. The wells are scanned for single clones within 3 weeks and the cells are transferred to 24-well plates with 1ml fresh media when the wells grow out. When they grow out they are then transferred to a T-25 flask. Amplification data indicated that the 4B3 and 4G2 masterwells were losing activity at higher MTX concentrations, so these cells
15 were frozen. For 1G2 and 3F6 the 8 clones (4 each from the 50 cells/well plates) were analyzed in a 6-day assay. None of the clones were significantly higher than the masterwell.

These clones were maintained while the amplification studies were being finished. Two of
20 the 1G2 clones showed decreased ASA production while the other two maintained their activity. One of the 3F6 clones was lost to contamination early on. One of the other 3F6 clones decreased its activity by half while the other two maintained their activity. None of the 3F6 clones totally lost their ASA production, so one of its clones (3F6-13C8) was chosen to go into second round cloning. This clone was chosen on the basis of cell growth,
25 viability, ASA activity and adaptability to spinner culture.

2nd Round Limiting Dilution Cloning

3F6-13C8 was cloned by seeding four 96-well plates each at 10, 5, 2.5 and 1.35 cells/well with 90% fresh media and 10% conditioned medium. This clone was chosen because it
30 retained its activity over time and adapted to spinner culture. The plates were fed 100 µl fresh media every 5-7 days. The cells were scanned at 4 weeks. Only 23 single clones were found. These were transferred to a 24 well plate and then to T-25 flasks as described above. Twelve grew out and were put into a 6-day assay. Two clones were chosen to be spinner adapted. They were selected on the basis of cell number, viability and ASA activity.
35 Clone 21 was twice as high and clone 22 was 30% higher than 3F6-13C8. They both were able to adapt to spinners. 3F6-13C8.21 was selected as the final clone since it had higher ASA activity.

Characterization of rhASA

The characterization of rhASA for specific ASA activity was done with partially purified material from the first round clones.

Purification

- 5 A preliminary purification of rhASA was attempted for the purposes of determining specific ASA activity.

1.2 liters of conditioned media from the first round clone, 3F6-13C8 (cultured for 10 days), were dialyzed against 20 mM Tris, pH 7.6 and loaded on a 50 ml DEAE-Sepharose column (AP-Biotech, Catalogue # 17-0709-01), equilibrated in the same buffer. A linear, 0-500 mM NaCl gradient was applied to the column, 5 ml fractions were collected and scanned by SDS-PAGE (Fig. 5). The contents of each lane of the gel is described in table 1 below.

Table 1. Contents of each lane in 4-12 % SDS PAGE. The [NaCl] indicates the salt concentration of the fractions.

Lane	Fraction	mM NaCl		Lane	Fraction	mM NaCl
1	Markers			14	Markers	
2	Start	0		15	41	140
3	Flow Thru	0		16	43	150
4	23	80		17	46	160
5	24	80		18	48	170
6	25	90		19	52	180
7	26	90		20	55	190
8	27	90		21	58	200
9	28	100		22	74	260
10	29	100		23	78	270
11	30	110		24	82	280
12	32	110				
13	37	130				

Fractions 45-54 were pooled to generate Pool A (55 ml). This had a specific activity of 9.5 units/mg at 0.17 mg/ml total protein. Fractions 55-62 were pooled to obtain Pool B (45 ml), which had a specific activity of 20.7 units/mg at 0.22 mg/ml total protein.

Conclusion

A rhASA production cell line (CHO DG44.42 subclone 3F6-13C8.21) was obtained after MTX amplification and two rounds of cloning. This clone has been designated "CHO-ASA 020409" and deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und

Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, GERMANY) for the purposes of patent deposit according to the Budapest Treaty on 6 June, 2002.

CHO cell-produced rhASA from a first round clone was subjected to DEAE-Sepharose
5 chromatography. The elution profile as determined by SDS-PAGE is shown in Fig. 5. The fractions were combined as two separate pools. Pool B (fractions 55-62), which had double the specific activity of Pool A still had other contaminating bands. Hence the actual specific activity is expected to be higher than the reported value of 20.7 U/mg. A total of 20 mg of total protein was recovered in pool A and B. Even if the purity is assumed to be only 50 %,
10 at least 10 mg of rhASA was obtained from 1.2 L of culture medium.

Example 3: In vitro characterisation of rhASA

Complete medium

MEM Eagle-Earles BSS with
15 15% FCS
2 mM Glutamine
2X conc. Vitamins
2X conc. non-essential AA
2X conc. MEM-AA
20 1 mM Na-pyruvate

TBS composition

10 mM Tris-HCl
150 mM NaCl
25 Adjust pH to 7.5

ASA activity assay protocol

ASA is able to catalyze the hydrolysis of the synthetic, chromogenic substrate, para-NitroCatechol Sulfate (pNCS). The product, para-NitroCatechol (pNC), absorbs light at
30 515nm. This principle is thoroughly described in the SOP for the method and here only briefly summarized.

2x ASA assay buffer

10 mM pNCS
35 10% (w/v) NaCl

1 mg/ml BSA
in 0.5 M NaAc pH 5.0

Stop solution

5 1M NaOH

Microtiter plate set-up

100 µl of sample at appropriate dilution (in TBS)

100 µl of 2x ASA assay buffer

10 The plate is incubated at 37° C for 30 minutes.

Stop by adding 100 µl stop buffer and read the plate at OD 515.

The ASA activity is then calculated using the millimolar extinction coefficient for the product in the formula:

15

$$(\text{OD } 515 \times V) / (12.4 \times t \times v) = \text{U/ml}$$

V = total volume for assay (ml)

t = incubation time (min)

20 v = sample volume (ml)

The millimolar extinction coefficient for para-NitroCatechol (pNC) = $12.4 \text{ M}^{-1} \text{ cm}^{-1}$

1 Unit of ASA activity is defined as the amount of enzyme that hydrolyses 1 µmol Para-NitroCatecholSulphate (pNCS)/min

25

BCA protein determination kit

Protein concentration was determined using the BCA Protein assay kit (no. 23225) from Pierce. This kit utilizes the principle of the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the Biuret reaction). The Cu^{+} ions are then reacted with a reagent containing
30 bicinchoninic acid resulting in a highly sensitive and selective colorimetric detection. The kit was performed according to the manual.

Microtiter plate set-up

10 µl of the BCA-Standards for a standard curve

35 10 µl of BSA reference in duplicate

10 µl of MilliQ water used as blank in at least triplicate

10 µl of sample or diluted sample in MilliQ water

200 µl of BCA assay buffer in all wells

Mix ~30 seconds

Incubate samples for 30 minutes at 37° C

Measure the absorbance within 10 minutes at 562 nm and determine the concentrations from the standard curve.

5 Introduction

At an early stage, before the purification process was developed, the rhASA was characterized in an *in vitro* cell system. For this purpose, ASA-containing supernatants from ASA-CHO cells were purified using two different purification methods. For the first experiments, enzyme from DG44.42 masterwells 3F6 and 1G2 cultivated in T-flasks was
10 purified on a ConA-Sepharose column which binds all glycosylated proteins. The column was equilibrated with 10 mM Tris-HCl, pH 7.0. After loading the sample, the column was washed with the equilibration buffer and then with 10 mM Tris-HCl + 1 M NaCl, pH 7.0. The bound proteins were eluted with 20 mM Tris-HCl + 0.5 M Mannose, pH 7.0 or 0.5 M Metyl-D-glucoside, pH 7.0. The enzyme was purified approximately 25 times and the
15 specific ASA activity in the eluate was around 5 U/mg.

To obtain a more pure enzyme preparation an affinity column where an ASA monoclonal antibody (mAb) 19-16-3 was bound to a PA-Sepharose matrix was used. This binding was chemically stabilized by Dimethyl pimelimidate dihydrochloride (DMP). ASA-containing
20 supernatants from T flasks with the DG44.42 subclone 3F6-13C8.21 were added to the column after equilibration with TBS, pH 7.4. After loading, the column was washed with the same buffer and then with TBS + 0.5 M NaCl, pH 7.4 and then again with TBS, pH 7.4. 10 column volumes were used for each washing step. The bound proteins were eluted using 2.8 M MgCl₂ in 0.1 M NaAc, pH 4.5. The enzyme was purified approximately 175
25 times and the specific ASA activity in the eluate was around 35 U/mg.

The ASA-containing eluates were used in the cell systems described below.

30 1. Cellular uptake of rhASA by MLD fibroblasts

Aim of the study

To get *in vitro* proof of concept regarding cellular uptake of rhASA in fibroblasts from MLD patients.

Experimental design

Fibroblasts from two different MLD patients (with the null mutation) with no endogenous ASA activity, GM00197 and GM00243 (purchased from Coriell Cell Repository, USA), were grown confluent in 6-well plates in complete medium (see appendix for details). Cells were washed with PBS and rhASA (ConA- or affinity-purified) was added (0-100 mU/ml) \pm 5 or 15 mM Mannose-6-Phosphate (M-6-P) or Glucose-6-Phosphate (G-6-P, control) in serum-free medium. The cells were left for 3 days and then harvested; medium was removed, centrifuged and buffer was changed to TBS using PD10 columns. Cells were washed with PBS and trypsinated, washed with TBS and then lysed with 0.5 % Triton X-100 in TBS. The lysed cells were centrifuged (13 200 rpm, 10 minutes) and lysate was collected. ASA activity and protein concentration were measured in all samples. The ASA activity was determined by the ASA activity assay described in the appendix below.

Results and discussion

The MLD fibroblasts had a low ASA background activity (close to zero). ConA-purified rhASA gave a maximal cellular load in GM00197 of 14 mU/mg total protein (after addition of 15 mU/ml medium for 3 days), see Fig. 6. The affinity-purified rhASA in the same cells gave a maximal load of 200 mU/mg total protein (14.5 times more load compared to addition of ConA-purified ASA), see Fig. 7. This was seen after addition of 100 mU/ml for 3 days. The affinity-purified preparation was more pure than the ConA-purified material (7 times higher specific activity in the affinity-purified material compared to the ConA-purified material). Addition of the latter (containing several glycosylated proteins) could result in a competition of the M-6-P receptors on the cell surface and therefore a slower cellular uptake. The uptake is rather linear over time as described in Experiment 3 and visualized in Fig. 11 and the maximal load only seems to be dependent on the enzyme dose given and the loading time. The cellular uptake of affinity-purified rhASA by the GM00243 fibroblasts was similar to the uptake seen by GM00197 (not shown). Addition of M-6-P (both 5 and 15 mM) at the same time as addition of rhASA totally blocked the cellular uptake of the enzyme while G-6-P had no effect. This suggests that rhASA specifically is taken up by the M-6-P receptor. Results from the experiment using addition of 5 mM M-6-P in MLD fibroblasts GM00243 is shown in Fig. 8. Adding 100 mU/ml rhASA, without adding M-6-P, 30-45 % of added enzyme was taken up after 3 days incubation time. 40 % of the added enzyme could be found in the medium (supernatant) at harvest (not shown). In total, 70 % of added enzyme could be recovered after 3 days incubation, indicating a good stability of the enzyme in the medium at 37 °C.

The ASA activity assay and protein determination were performed in micro-titer plate as briefly described in the appendix. See also appendix for description of complete medium composition.

2. Staining of intracellular rhASA in MLD fibroblasts loaded in vitro

Aim of the study

To get proof of concept for lysosomal uptake of rhASA *in vitro*.

5

Experimental design

- Fibroblasts from a MLD patient (GM00243) were grown almost confluent in complete medium on 5 glass slides (Falcon). Cells were washed and affinity-purified rhASA was added in serum-free medium (0 or 100 mU/ml) to 3 slides. 5 mM M-6-P was added to one of these. The cells were left for 3 days and then harvested; medium was removed, cells were washed with PBS and then fixated in 3% Paraformaldehyde (PFA) in PBS for 1 hour at 37° C. Cells were then treated with 50 mM NH₄Cl in PBS to neutralize formic acid formed by PFA oxidation and thereafter permeabilized in 0.3 % Triton X-100 in PBS for exactly 2 x 5 minutes. Cells were then incubated with 0.2 % BSA in PBS for 3 x 5 minutes.
- 10 of these. The cells were left for 3 days and then harvested; medium was removed, cells were washed with PBS and then fixated in 3% Paraformaldehyde (PFA) in PBS for 1 hour at 37° C. Cells were then treated with 50 mM NH₄Cl in PBS to neutralize formic acid formed by PFA oxidation and thereafter permeabilized in 0.3 % Triton X-100 in PBS for exactly 2 x 5 minutes. Cells were then incubated with 0.2 % BSA in PBS for 3 x 5 minutes.
- 15 Cells were stained for 1 h 45 min with a green lysosomal marker (Lysosomal Associated Membrane Protein-1-FITC, LAMP-1-FITC) and the ASA mAb 19-16-3 plus red RAM-TRITC (DAKO) which gives a red fluorescence. Cells were stained with 0.2 % BSA in PBS and RAM-TRITC for background controls.
- 20 The double stained cells were incubated with Normal Mouse Serum (NMS, 1:100) for 10 minutes between first and second color. The slides were mounted and saved in -20° C before analyzed using fluorescence microscopy.

- Staining was performed in dark, in a humidity chamber and cells were washed between each step with PBS or 0.2 % BSA in PBS 2 x 5 minutes.
- 25

Results and discussion

- Controls were negative (not shown) and loaded cells double stained with LAMP-1-FITC (green fluorescence) and ASA mAb 19-16-3 + RAM-TRITC (red fluorescence) showed a lysosomal pattern. If the two pictures were overlaid, co-localization could be seen in yellow/orange. There was some green and red color in this over-lay picture, but most of the coloring overlapped and showed the yellow/orange color, see Fig. 9. This suggests a lysosomal localization of the added rhASA.
- 30

35

3. Kinetics on cellular uptake of rhASA by MLD fibroblasts *in vitro*

Aim of the study

- 5 To see the kinetics by which rhASA is taken up by MLD fibroblasts *in vitro*.

Experimental design

Fibroblasts from a MLD patient (GM00243) were grown confluent in 6-well plates in complete medium. Cells were washed with PBS and affinity-purified rhASA was added (0 or
10 100 mU/ml) in complete medium with or without Fetal Calf Serum (FCS). The cells were left for 1, 3, 5 and 8 hours resp. In another set-up, cells were left for 15, 20, 40, 65 and 69 hours resp. At these time-points, supernatant was removed, centrifuged and buffer was changed to TBS using PD10 columns. Cells were washed with PBS and trypsinated, washed with TBS and then lysed with 0.5 % Triton X-100 in TBS. The lysed cells were centrifuged
15 (13 200 rpm, 10 minutes) and lysate was collected. ASA activity and protein concentration were measured in all samples (see appendix for details).

Results and discussion

A cellular uptake could be measured already after 1 hour incubation with the enzyme and a
20 linear uptake could be seen for up to 69 hours. The uptake is probably dependent on M-6-P receptor traffic between the cell surface and the lysosomes. If rhASA is present outside the cells, the receptors will bring the enzyme to the lysosomes. After 69 hours, cells were loaded with 240 mU/mg total protein. The intracellular rhASA load after cell feeding in the presence of FCS was higher (1.1-2 times) compared to the load after feeding in serum-free
25 medium. This may be due to a higher cell metabolism (faster M-6-P receptor traffic) when FCS is present or a serum factor dependent increased affinity of the enzyme for the receptor. After 2-3 hours incubation with the enzyme (100 mU/ml), the intracellular content was equal to the levels found in non-affected fibroblasts (10-15 mU/mg total protein, not shown). For results, see Fig. 10 and 11.

30

Conclusion

Addition of rhASA to culture medium of fibroblasts of a MLD-affected patient resulted in enzyme activities comparable to those seen in normal fibroblasts. The uptake was inhibited when 5 mM M-6-P was included in the medium. This shows that rhASA is taken up by
35 binding to the M-6-P receptors on the cells. From the data shown in Fig. 6 and 7 it is seen the full restoration of activity (10-15 mU/mg) occurs at 5 - 15 mU/ml rhASA in the medium depending on the purity.

4. Intracellular stability of rhASA in MLD fibroblasts loaded with rhASA *in vitro*

Aim of the study

- 5 To see for how long time intracellular rhASA is stabile in MLD fibroblasts after loading *in vitro*.

Experimental design

- Fibroblasts from a MLD patient (GM00243) were grown confluent in T25 flasks in complete medium. Cells were washed and affinity-purified rhASA was added (0 or 100 mU/ml) in complete medium. The cells were left for 24 hours after which supernatant was removed, cells were washed with PBS and trypsinated. Half of the cells were kept in culture (without rhASA) and the other half was washed with TBS and then lysed with 0.5 % Triton X-100 in TBS. The lysed cells were centrifuged (13 200 rpm, 10 minutes) and lysate was collected.
- 15 After 48 more hours, the cells were confluent and subcultivated with a split ratio of 1:5. Again, cells that were not used for further cultivation were washed and lysed as described above. After 72 hours, the cells were again confluent (120 hours after rhASA withdrawal). They were washed, trypsinated and lysed. All samples were kept frozen before analyzed for protein content and ASA activity (see appendix for details).

20

Results and discussion

- 24 hours load with 100 mU/ml rhASA resulted in an intracellular ASA activity of 170 mU/mg (time zero). 48 hours after ASA withdrawal and subcultivation (1:2), 100 mU/mg was found inside the cells (approximately 1:2 the activity at time 0). After 72 more hours, and a 1:5 subcultivation, 24 mU/mg was measured (approximately 1:5 the activity at time 48 h). See Fig. 12 for results. The conclusion is that rhASA is taken up by the cells and once intracellular, the enzyme is stabile at least for 120 hours (5 days).
- 25

Example 4: Small-scale cultivation of producer cell

30 Cell line and culture medium

- A DHFR minus Chinese Hamster Ovary (CHO) cell line, DG44, expressing rhASA (DG44.42 subclone 3F6-13C8.21), is routinely cultivated in modified EX-CELL 302 CHO serum-free medium without phenol red (JHR Biosciences Europe, UK). The medium contains 1.6 g/L sodium bicarbonate, 4 mM HEPES and 0.1 % Pluronic®F-68 and is supplemented with 4 mM L-Glutamine and 3.4 g/L D-(+)-Glucose. The pH of the medium is 7.0-7.4.
- 35

Selection is maintained by using 20 nM methotrexate (MTX), a competitive inhibitor of DHFR. 1 mL of a 20 μ M MTX working solution is added to 1 L of complete medium.

No antibiotics are used. Samples for mycoplasma test are obtained periodically and analysed by the Mycoplasma Laboratory at the National Veterinary Institute, Uppsala, Sweden. All samples investigated were found to be negative for mycoplasma.

T-flasks and shake flasks

T-flasks (175 cm²) with 60 mL medium are seeded with 2.5-3 x10⁵ cells/mL and incubated at 37 °C and 5 % CO₂ in a humidified incubator for 72-96 hours. After 3-4 days, a cell concentration of 7 x10⁵ – 1 x10⁶ cells/mL can be expected. Corresponding ASA activity in the medium is 0.15 U/mL. These cells are then used to inoculate 1 L plastic Erlenmeyer culture shake flasks containing 150-200 mL of medium to a cell density of 2.5-4 x10⁵ cells/mL. The shake flasks are sealed with plug seal caps, open for gas exchange, and incubated 72-100 hours on a rotary shaker (80 rpm) at 37 °C and 5% CO₂. Cell growth is followed by withdrawing samples from the cultures at different time-points and counting viable and dead cells in a haemocytometer using the trypan blue exclusion method. A typical growth curve is seen in Figure 13. At time for harvest, normally 80-100 hours post inoculation, the cell suspensions, having cell densities at 1.3-1.9 x10⁶ cells/mL and cell viabilities of 75-90 %, are centrifuged (110g, 8 min) in sterile tubes in a swing-out centrifuge (Eppendorf) at 20 °C. The collected supernatants are filtered (0.45 μ m) and passed on to the purification process or stored frozen (-20 °C) for later use. Fresh and filtered supernatant from shake flasks holds an ASA activity of 0.25 U/mL. The cell pellets are resuspended in fresh medium at RT to a suitable cell density and passaged to new shake flask cultures.

25

Currently, MTX is not used in shake flask cultures. An experiment, concerning the stability of ASA production was carried out. Two shake flask cultures, one with 20 nM MTX and the other without MTX, were run for several weeks. There was no difference looking at ASA production in the two cultures, see Figure 14. This indicates that DG44.42 subclone 3F6-13C8.21 is a stable clone, which does not require constant MTX selection for high ASA production, at least for the time explored. Long-time stored cells frozen in liquid nitrogen in the working cell bank are still under MTX selection. New vials of cells are thawed regularly, at least every fifth month, and the ASA production is checked every week during the cultivation process in progress.

35

Example 5: Semi-large scale fermentation

Based on the small scale experiment results (T-flasks, shake and spinner flasks), bioreactor cultivations were designed to grow CHO-ASA cell line (DG44.42 subclone 3F6-13C8.21) in order to produce the secreted ASA protein.

The equipment used – from B.Braun - included 5 liter working volume bench-top bioreactors controlled by a DCU-3 controller and MFCS software (B.Braun Biotech, Cat no. 8877017) for data collection.

- 10 The starting material is a CHO-ASA cell bank (DG44.42 subclone 3F6-13C8.21) performed from spinner flasks.

The medium is a modified and serum-free EX-CELL 302 formulation from JRH Biosciences supplemented with 4 mM glutamine and with 1 to 4 g/l of glucose, see example 2.

15

The cells can be either diluted or centrifuged before inoculation into the bioreactor. The seeding densities tested are 2 and 5×10^5 cells/ml. The main set-points of the bioreactor culture are:

- 20 Table 2: parameters and set-points for bioreactor culture

Parameters	Set-point
agitation	100 rising to 160 rpm
temperature	37°C
pH ⁽¹⁾	7.2+/-0.2
dissolved oxygen ⁽²⁾	30%

⁽¹⁾ pH is controlled by the addition of sodium hydroxide or CO₂ sparging.

⁽²⁾ Dissolved oxygen is controlled by sparging air + oxygen.

- 25 2 batches were produced, this first with 2.5 liters and the second one with 5 liters. The main results are presented in the following table 3:

Table 3: main results of batch cultivation for ASA production

Parameters	First batch	Second batch
Volume (liter)	2.5	5
Initial cell density (cell/ml)	2×10^5	5×10^5
Maximum cell density (cell/ml)	3.3×10^6	3.8×10^6
ASA production (U/liter)	1.19	1.36
ASA production per 10^6 cells (U/ 10^6 cells)	0.36	0.36
Estimated ASA production (mg/liter)	24	27
ASA production per 10^6 cells (mg/ 10^6 cells)	7.2	7.1

The main achievements are:

5

We obtained a single cell suspension culture, even at the end of the cell growth when cell densities went above 3×10^6 cells/ml.

We obtained a constant ASA production per cell, 0.36 U/ 10^6 cells or around 7 mg/ 10^6 cells.

10 This specific ASA production is also higher compared to those obtained in spinner flasks (average: 0.26 U/ml).

The results obtained from these two batches are comparable both regarding cell growth and ASA production (see figure 15 and 16 below).

15

The overall product yield was 60 mg for the first batch and 135 mg for the second one.

The following tables (4 and 5) summarise the parameters followed during the 2 batches.

20 ASA activity was measured according to the materials and methods in example 3.

Table 4: main parameters of batch #1

Time (hour)	Cell density (cell/ml)	Viability (%)	Doubling time (hour)	Glucose (g/l)	Lactate (g/l)	NH4+ (mM)	ASAU/ml
0	2.00E+05	83		3.11	0	1.41	
20	2.00E+05	77	N/A	2.97	0.15	1.83	
41	3.60E+05	84	25h	2.62	0.46	2.56	
65	9.30E+05	85	18h	2.35	0.87	3.39	0.14
74	1.20E+06	93	24h	2.13	0.74	3.83	0.19
91	1.50E+06	88	53h	1.7	0.92	4.47	0.27
116	2.50E+06	89	34h	1.01	1.23	4.89	0.47
142	3.10E+06	87		1.13	1.79	6.98	0.64
163	3.30E+06	87		0.13	2.07	7.59	0.83

see also Fig 15 upper curve and Fig 16 upper curve.

5

Table 5: main parameters of batch #2

Time (hour)	Cell density (cell/ml)	Viability (%)	Doubling time (hour)	Glucose (g/l)	Lactate (g/l)	NH4+ (mM)	ASAU/ml
0	5.00E+05	92		3.02	0.31	2.44	
25	7.60E+05	88	41.4	2.17	0.98	3.12	
44	1.10E+06	89	35.6	1.5	1.47	3.59	
69	2.30E+06	88	23.5	0.28	2.18	4.56	
91	2.70E+06	90	95.1	0.91	2.58	5.41	0.67
97.5	2.90E+06	90	63.0	2.06	2.97	5.54	#N/A
127.5	3.80E+06	93	76.9	0.01	3.94	6.02	1.06
147	3.10E+06	81		1.24	4.21	6.89	1.15
163	3.10E+06	82		0.7	3.94	7	1.36

see also Fig 15 lower curve and Fig 16 lower curve.

Example 6: Partially developed purification scheme, formulation, filling and lyophilization

Aim: To develop a purification process for rhASA in 20-200 ml scale intended for scale-up to large scale production. The quality and purity of the final product (rhASA) should very high and according to the specifications (approved for clinical trials). The process will include a capture step, 1-2 intermediate purification steps, 1 polishing step, 1-2 virus removal steps and 1 formulation step. 1 or more buffer exchange steps will also be included (Diafiltration). The small scale process should be transferred to intermediate and finally large-scale production.

10

Experimental design: Several different chromatography gels will be tested and performance of the different steps will be analysed by a battery of analytical methods described briefly below:

- | | | |
|----|-------------------------------------|-------------------------------------|
| 15 | <u>Enzyme activity:</u> | Arylsulfatase assay (see example 3) |
| | <u>Total protein concentration:</u> | BCA analysis |
| | <u>rhASA concentration:</u> | rhASA ELISA |
| | <u>Purity:</u> | HPLC and SDS-PAGE |
| | <u>Identity:</u> | HPLC |
| 20 | <u>HCP proteins:</u> | ELISA |
| | <u>Endotoxin level:</u> | Outsource to contract Lab |

Outline of purification process in 20 ml column scale

25 **Step 1: Concentration/Diafiltration**

Spinner (activity: 0.2 – 0.5 U/ml; protein conc 0.2 – 0.5 mg/ml) or Bioreactor (activity: 1.0 – 2.0 U/ml; protein conc 1.0 – 2.0 mg/ml) produced media with expressed rhASA was concentrated approximately 10x for Spinner and 2-10x for Bioreactor produced media using Tangential flow filtration (TFF) against a Pellicon Biomax polysulphone filter with 30 kDa cut off.

Example: 1 litre spinner produced media was concentrated using TFF against a 50 cm² Biomax 30 kDa filter to 100 ml. Transmembrane pressure (TMP) was 25 (Pin = 30 psi; Pout = 20 psi). 100 ml of 2 M NaCl in 20 mM Tris-HCl pH 7.5 was added. Solution was stirred for 10 minutes and concentrated to 100 ml again.

After that, diafiltration against 4 volumes of 20 mM Tris-HCl, pH 7.5 with TMP of 25 was applied. Specific activity of sample was 0.5 – 1.5 U/mg. Yield 90 – 100%.

Step 2: Capture step – DEAE sepharose FF

Sample from step 1 was applied on a 20 ml DEAE sepharose packed in a 16 mm diameter column (Pharmacia XK 16) equilibrated with 20 mM Tris-HCl pH 7.5 (referred to as standard buffer). Flow rate was 4 ml/min. Protein bound to the DEAE gel was then washed with 2-4 column volumes (CV) of standard buffer followed by 2-4 CV's of 0.1 M NaCl in standard buffer. RhASA was eluted with 3-5 CV's of 0.3 M NaCl in standard buffer. Fractions containing rhASA activity was pooled (specific activity: 3.5 U/mg) and used for further purification. Yield 90 – 100 %.

10

Step 3: Intermediate step 1 – Octyl Sepharose FF

Sample pool from step 2 was mixed 1:1 with 1.0 M Na₂SO₄ and applied on a 20 ml octyl sepharose FF packed in a 16 mm diameter column (Pharmacia XK 16) equilibrated with standard buffer + 0.5 M Na₂SO₄. Flow rate was 4 ml/min. Column was washed with 2-4 CV's of the same buffer. RhASA was eluted with standard buffer and fractions containing activity was pooled (specific activity 20 – 25 U/mg) and used for further purification. Yield 90 – 100 %.

Step 4: Intermediate step 2

Several options have been tested and the possibilities at this stage are listed below.

Presently preferred step at this point is Macro prep, ceramic hydroxyapatite type II :

1. Macro prep, Ceramic hydroxyapatite type II, 40 µm.

Brief description: Equilibrate column (Gel volume = 20 ml) with 4-6 CV's of 10 mM Tris-HCl pH 7.5 (Buffer A). Flow rate 2-5 ml/min. Buffer B = 400 mM Sodium Phosphate . Load sample from step 3 on column. Wash with 2-4 CV's of Buffer A. Elute rhASA with 75 % A + 25 % B buffer. Collect peak containing rhASA activity. Specific activity 25 – 35 U/ mg. Wash column with 2-4 CV's 100 % B. Yield not defined yet (tentative: 80 – 100 %).

2. Blue Sepharose FF

Equilibrate column with 5-8 CV's of 20 mM Sodium acetate pH 4.5. Buffer exchange sample from step 3 using diafiltration (TFF) against a 50 cm³ Biomax 30 kDa filter into 20 mM Sodium acetate pH 4.5. Load sample onto column. Wash with 2-4 CV's of 20 mM Sodium acetate pH 4.5. Elute protein with 20 mM Tris-HCl pH 7.5. Collect fractions containing rhASA activity. Specific activity 20 – 30 U/mg.

35 3. Source 15 Q – anion exchanger

Description of parameters: see Step 5

4. Source 15 S – cation exchanger

Step 5: Polishing step

Several options have been tested and the possibilities at this stage are listed below.

Presently preferred step at this point is Source 15 Q:

1. Macro prep, Ceramic hydroxyapatite type II, 40 µm.
- 5 Description of parameters: see Step 4
2. Source 15 Q – anion exchanger
 Equilibrate column with 4 CV's of 200 mM Tris-HCl pH 7.5. Change to 5 CV's of 20 mM Tris-HCl pH 7.5 (standard buffer). Flow rate: 2- 5 ml/min. Load sample from step 4 (should be in standard buffer before application) onto column. Wash with 2-4
 10 CV's of standard buffer. Apply a shallow gradient from 0 to 100% of 1 M NaCl in standard buffer (flow rate 2 ml/min, gradient time 50 minutes). Collect fractions containing rhASA activity. Tentative specific activity 30 – 50 U/mg. Yield not defined yet (tentative: 80 – 100 %).
3. Source 15 S – cation exchanger
 15 No information on parameters yet but should run in acidic pH. Suggestion:
 Equilibration buffer = 20 mM Sodium Acetate pH 4.5. Elute with a NaCl (increasing salt concentration) or pH (increasing pH) gradient.

Step 6: Virus filtration step

- 20 Virus filtration is performed on the product pool from step 5 using a Viresolve NFP filter from Millipore with an applied pressure of 20 – 50 psi.

Step 7: Diafiltration / Formulation step

- Tangential flow filtration (TFF) against a Pellicon Biomax polysulphone filter with 30 kDa
 25 cut off against 5-10 x Volumes of formulation buffer is performed. Two formulation buffers is tested:

Formulation buffer 1.

- | | | |
|----|----------------------------------|--------------|
| | Na ₂ HPO ₄ | 3.10-3.50 mM |
| 30 | NaH ₂ PO ₄ | 0.4-0.6 mM |
| | Glycine | 25-30 mM |
| | Mannitol | 220-250 mM |
| | Water for injection (WFI) | |

Formulation buffer 2.

Sodium Citrate	4.0-5.0 mM
Citric Acid	0.3-0.8 mM
Mannitol	200-250 mM
5 Tween 80	3.0-5.0 mM
Water for injection (WFI)	

The pH and osmolality in both Formulation buffers is balanced to 7.5 ± 0.2 and 300 ± 50 mOsm/kg respectively. Final protein concentration is according to the specification (>5
10 mg/ml).

Step 8: Formulation, Filling and Freeze-drying

Formulation and dosage form

In the development of dosage form the stability of rhASA is focused. The development
15 process starts with an aqueous solution and will, most likely, end up as a freeze-dried product.

Two different formulations are tested: Formulation buffer 1 and Formulation buffer 2, see Step 7.

20

Both these formulations are known to stabilize proteins in aqueous solutions as well as in freeze-dried powders. The pH and osmolality in both Formulation buffers are balanced to 7.5 ± 0.2 and 300 ± 50 mOsm/kg respectively. Final protein concentration should be according to the specification and in the range 4-10 mg/ml.

25

A freeze-dried product of rhASA is produced at a production unit according to EU GMP practice. The filling and freeze-drying is performed in a room classified as Class A. During production the filling zone is monitored with particle count and settle plates. The personnel are regularly trained according to EU GMP and monitored after each production with glove
30 prints. The sterility of equipment and materials are secured by validated sterilization procedures.

Filling

The bulk drug substance of rhASA are aseptically filled in sterile type I glass vials.

Freeze-drying

35 The vials are freeze-dried with freeze-drying cycles specifically developed for rhASA in the two different formulations described above. Nitrogen gas is filled into the vials in the end of the cycle and eventually closed with stoppers and capped. The batch is finally analyzed and released according to the specification.

Conclusion

A large scale purification scheme has been worked out. Also information on the further processing of the product including the formulation, filling and freeze-drying is provided.

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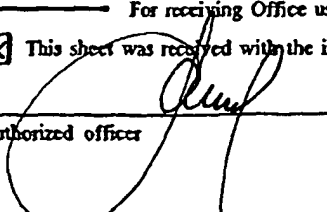
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Applicant's or agent's file reference	23201PC01	International application No.	2002
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**INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL**

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>11</u> , line <u>18-19</u> .	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	
Address of depositary institution (including postal code and country) Maschrodenweg 1b D-38124 Braunschweig Federal Republic of Germany	
Date of deposit 6 June 2002	Accession Number DSM ACC 2550
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
As regards the respective Patent Offices of the respective designated states, the applicant requests that a sample of the deposited microorganism only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau late (specify the general nature of the indications e.g. "Accession Number of Deposit")	

<p>For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer </p>	<p>For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>
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CLAIMS

1. A cell capable of producing recombinant human ASA, said cell comprising the DNA fragment shown in SEQ ID NO 1.
- 5 2. A cell according to claim 1 comprising the 1578 basepair *EcoRI* - *XbaI* fragment of the DNA fragment shown in SEQ ID NO 2.
3. A cell according to claim 1 or 2 obtained by use of the expression plasmid pAsaExp1
10 having SEQ ID NO:2.
4. A cell according to any of claims 1 - 3 obtained by transfection of a non-human mammalian cell line.
- 15 5. A cell according to claim 4 obtained by transfection of chinese hamster ovary (CHO) cells.
6. A cell according to any of claims 1 - 5 obtained by the culture of the human ASA production cell line CHO DG44.42 subclone 3F6-13C8.21 which has been deposited at the
20 DSMZ for the purposes of patent deposit according to the Budapest Treaty on 6 June 2002.
7. A method for the preparation of recombinant human ASA, the method comprising
 - a) introducing, into a suitable vector, a nucleic acid fragment comprising the DNA fragment
25 shown in SEQ ID NO 1;
 - b) transforming a cell with the vector obtained in step a);
 - c) culturing the transformed host cell under conditions facilitating expression of the nucleic
30 acid sequence;
 - d) recovering the expression product from the culture.
8. A method according to claim 7 further comprising a fermentation step.
- 35 9. A method according to claim 7 or 8 further comprising a purification step.
10. A method according to any of claims 7-9, wherein the ASA is recombinant human ASA encoded by SEQ ID NO 1.

11. An expression plasmid pAsaExp1 as shown in SEQ ID NO 2 for use in the expression of rhASA in cells.

5 12. A rhASA produced by the method of any of claims 7-10.

13. Use of the rhASA according to claim 12 for the preparation of a medicament for the treatment of Metachromatic Leukodystrophy.

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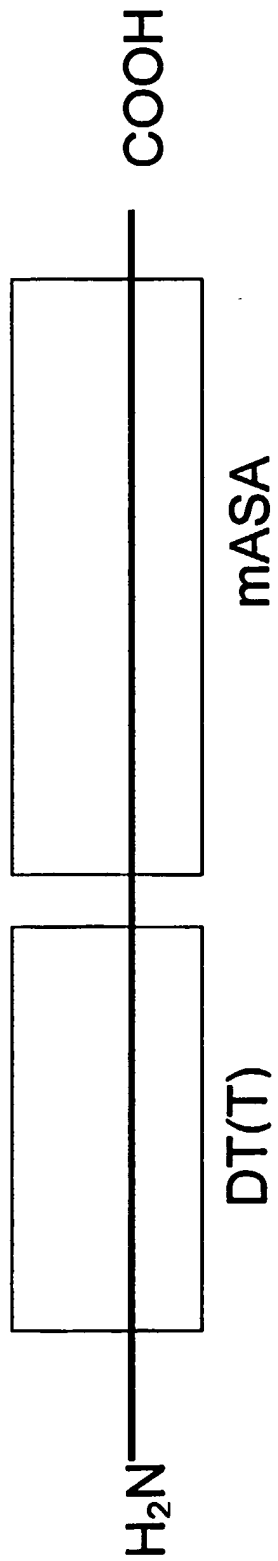


Fig. 1

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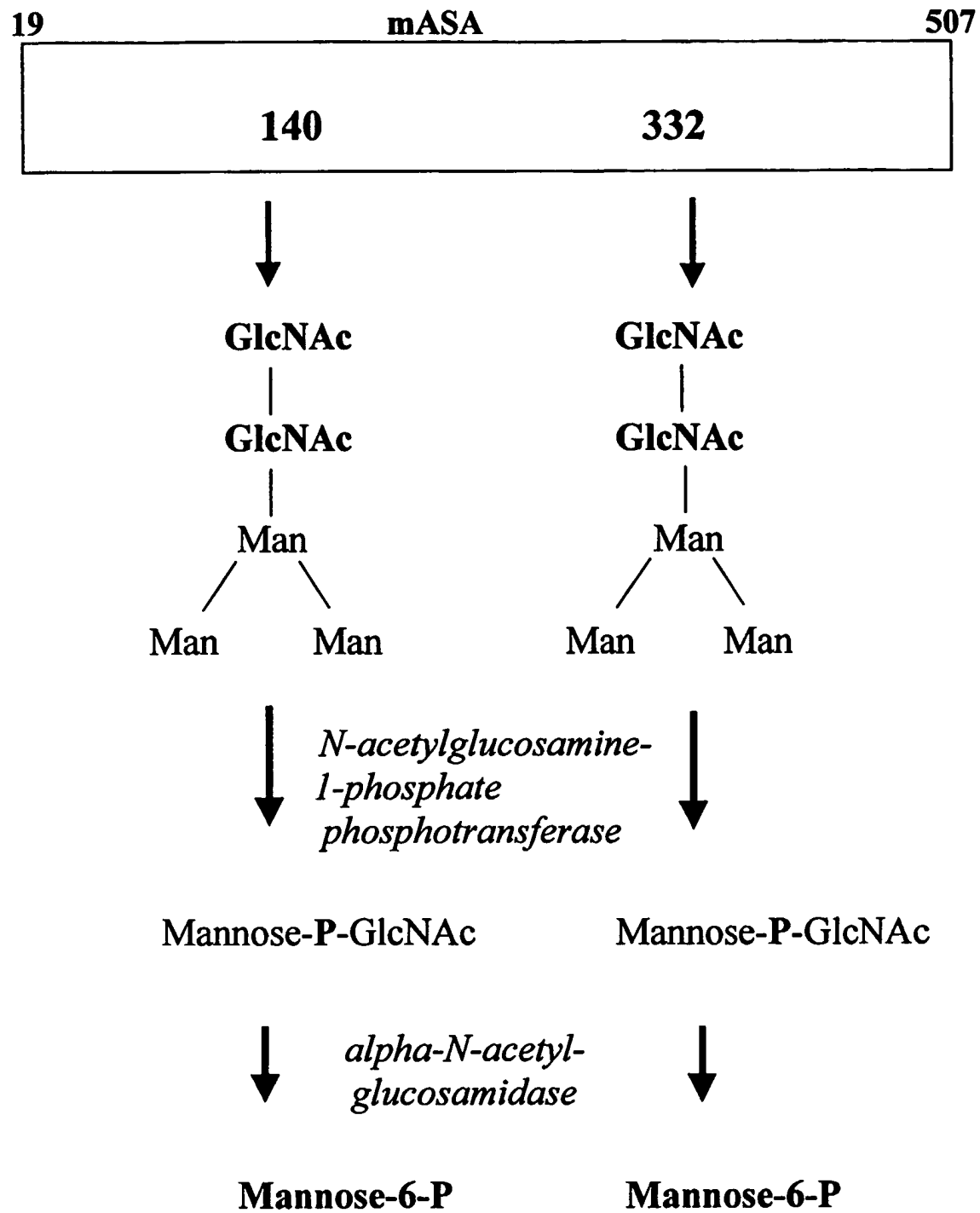


Fig. 2

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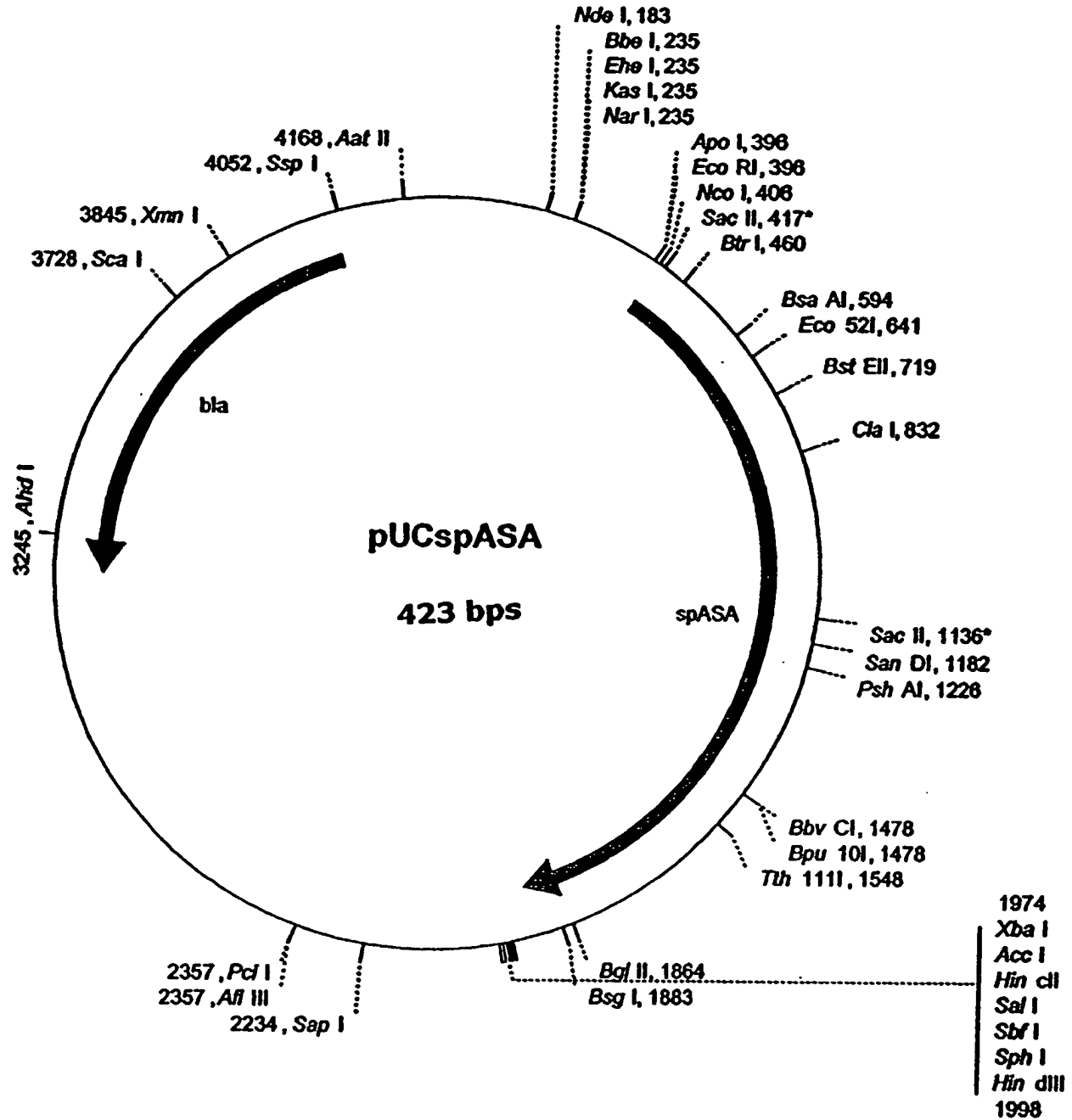


Fig. 3

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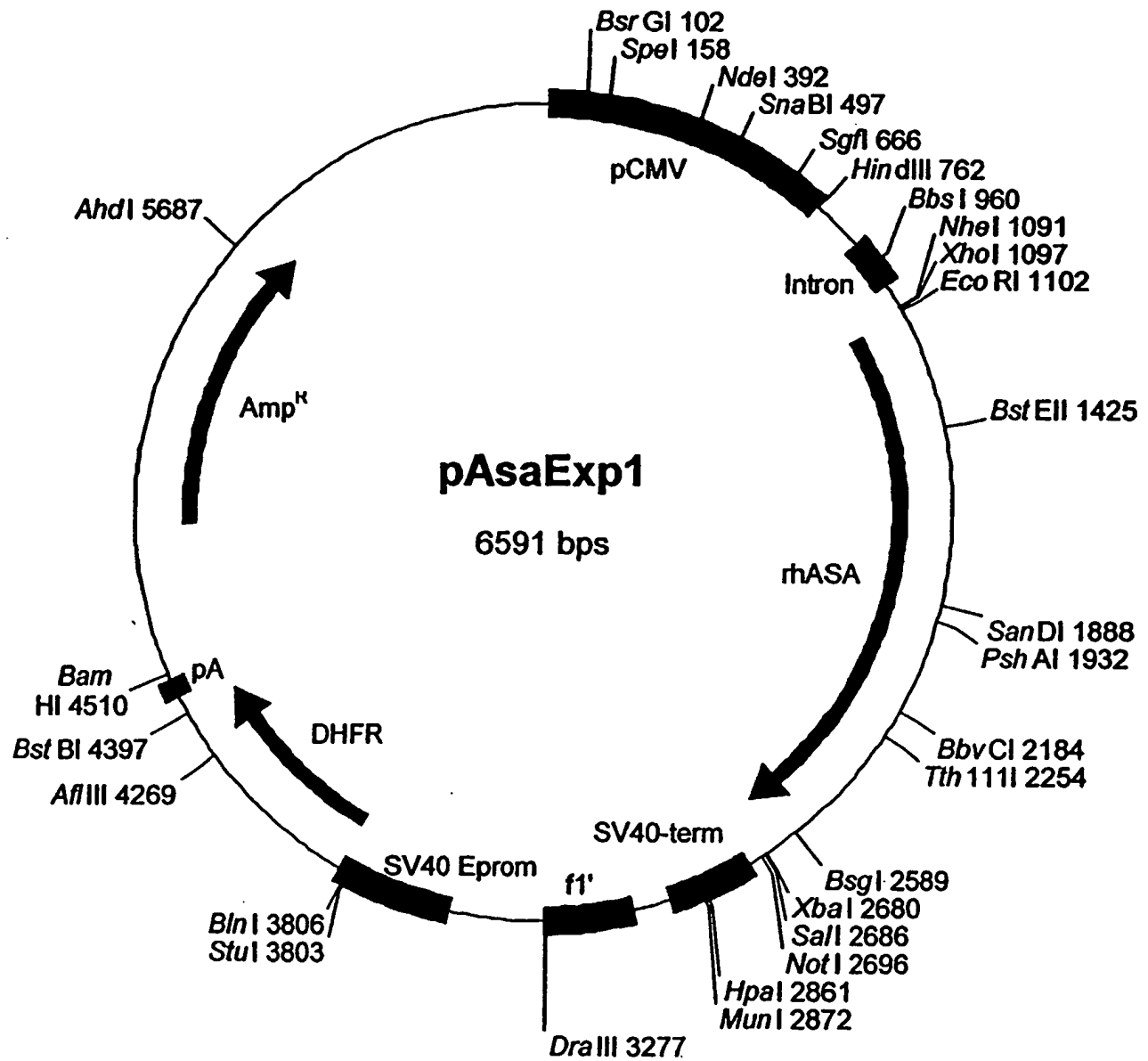


Fig. 4

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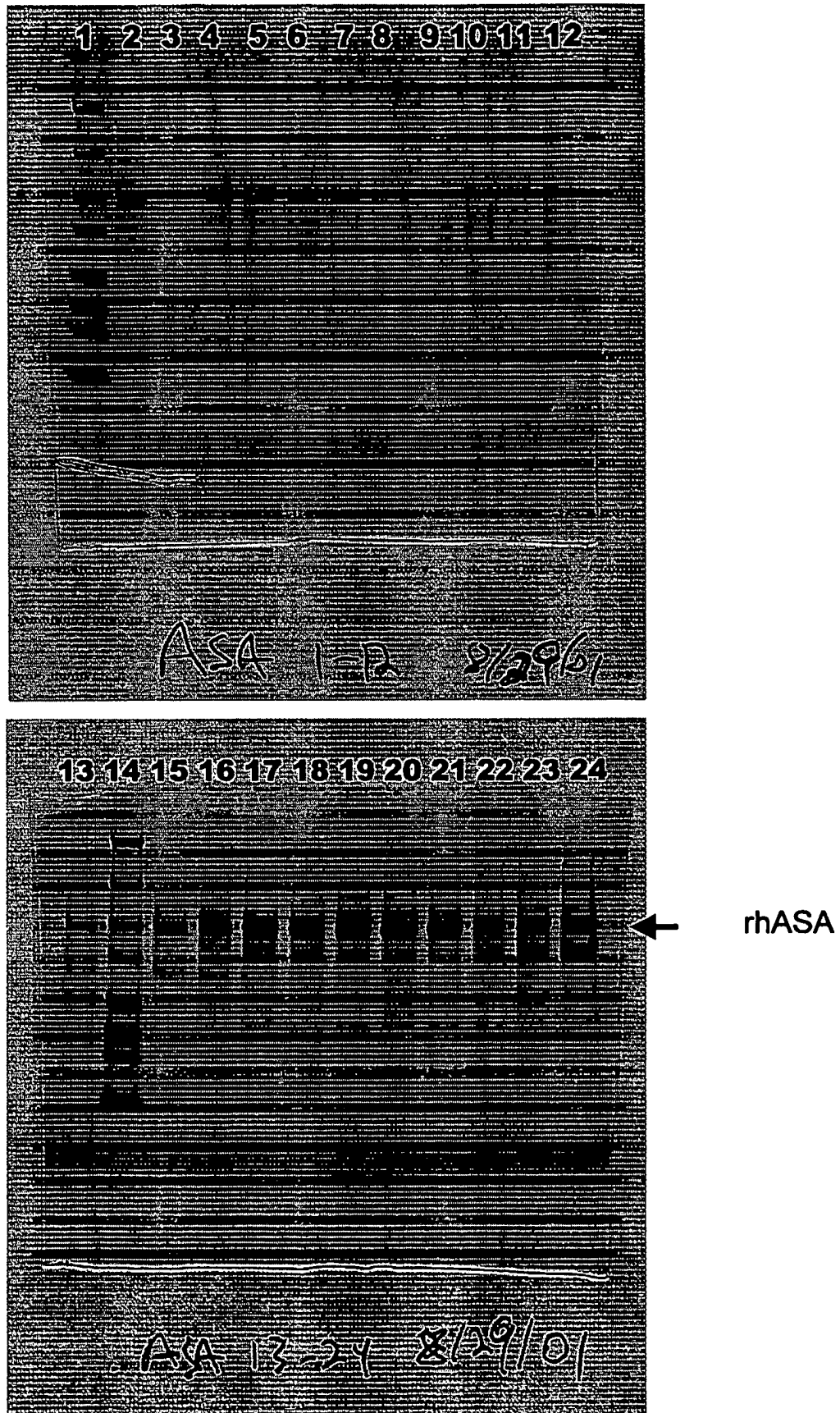


Fig. 5

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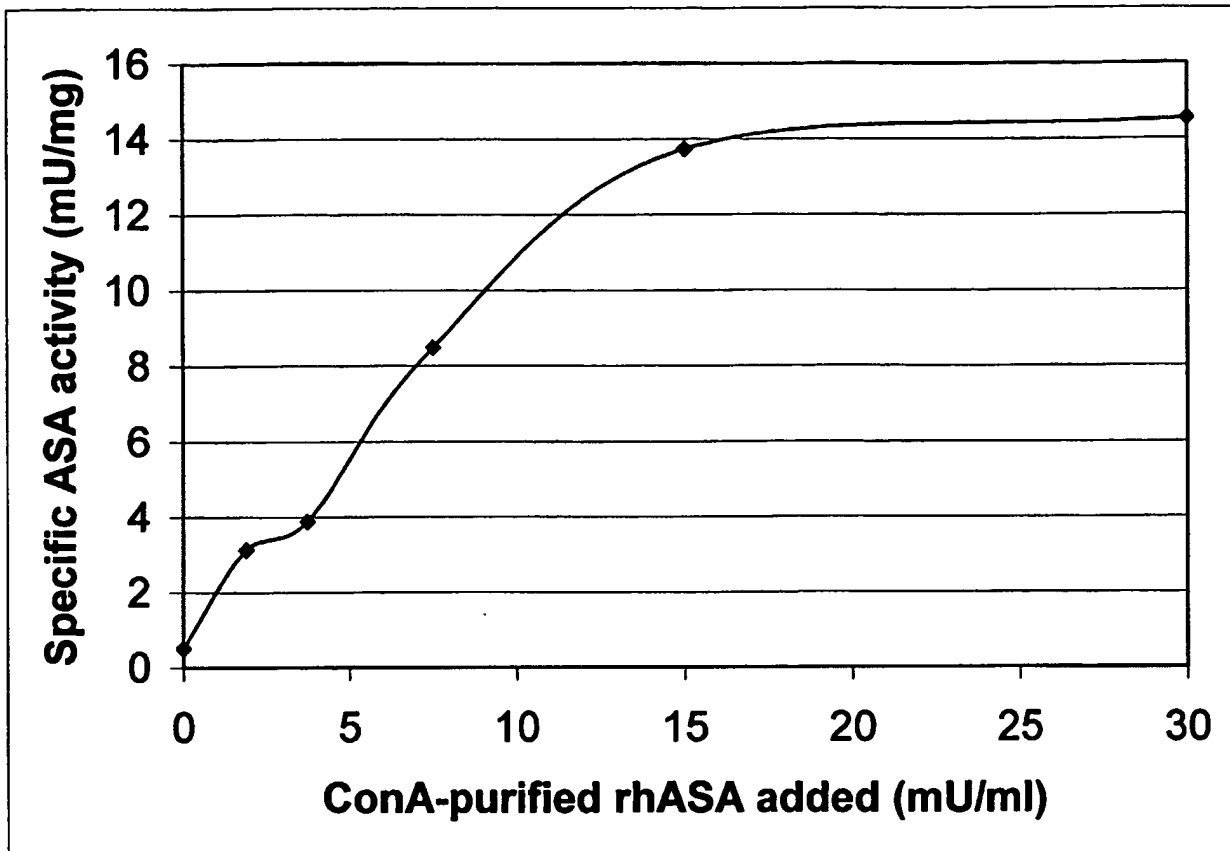


Fig. 6

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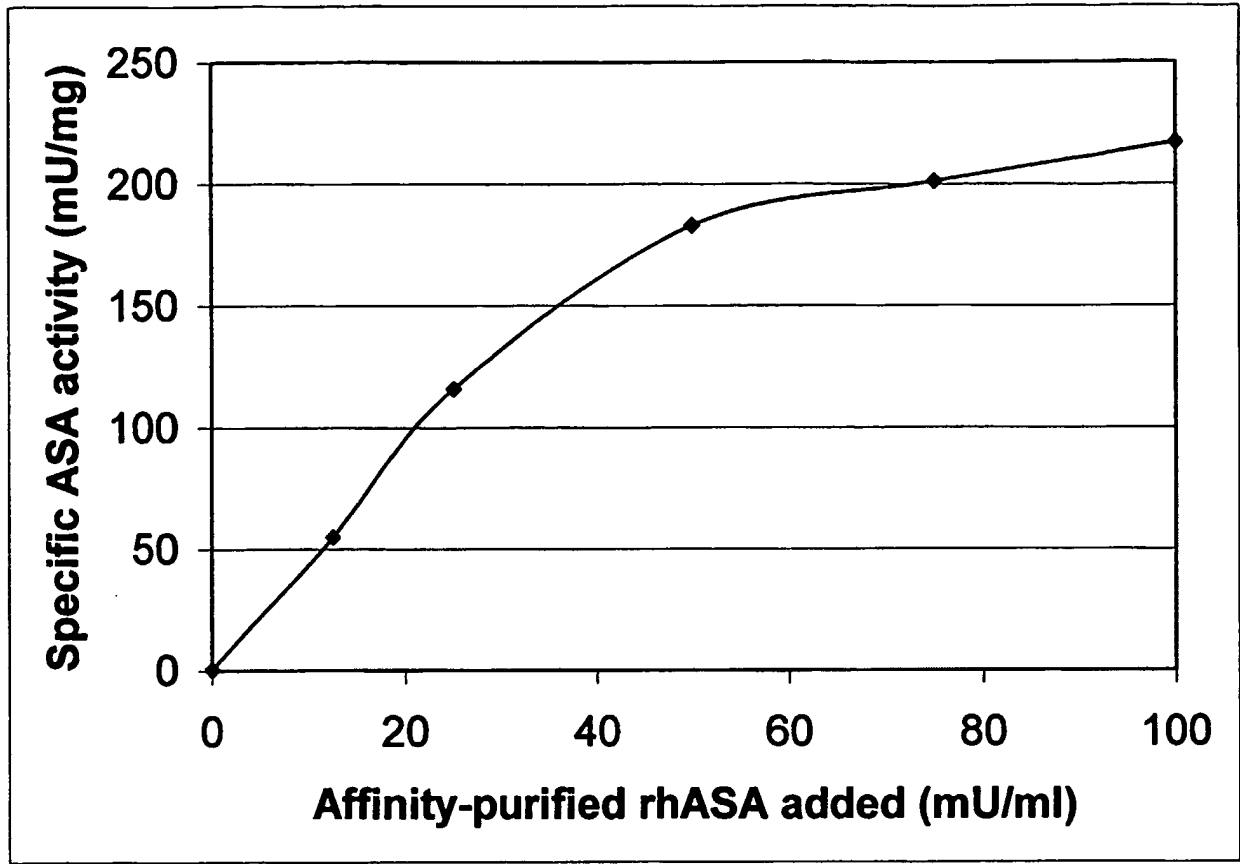


Fig. 7

8/16

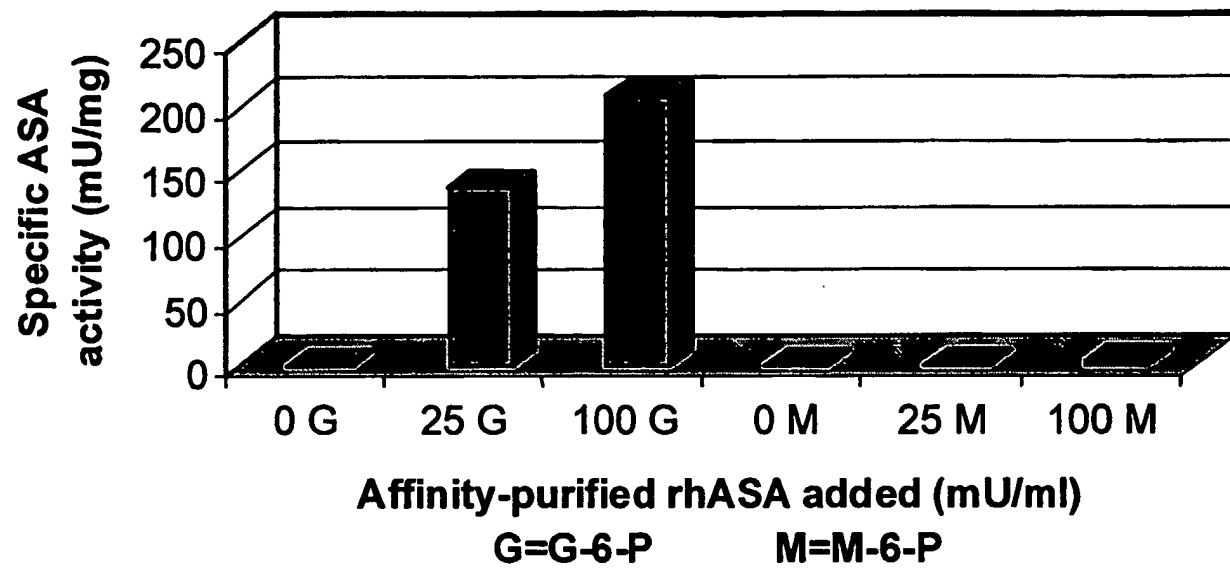


Fig. 8

9/16

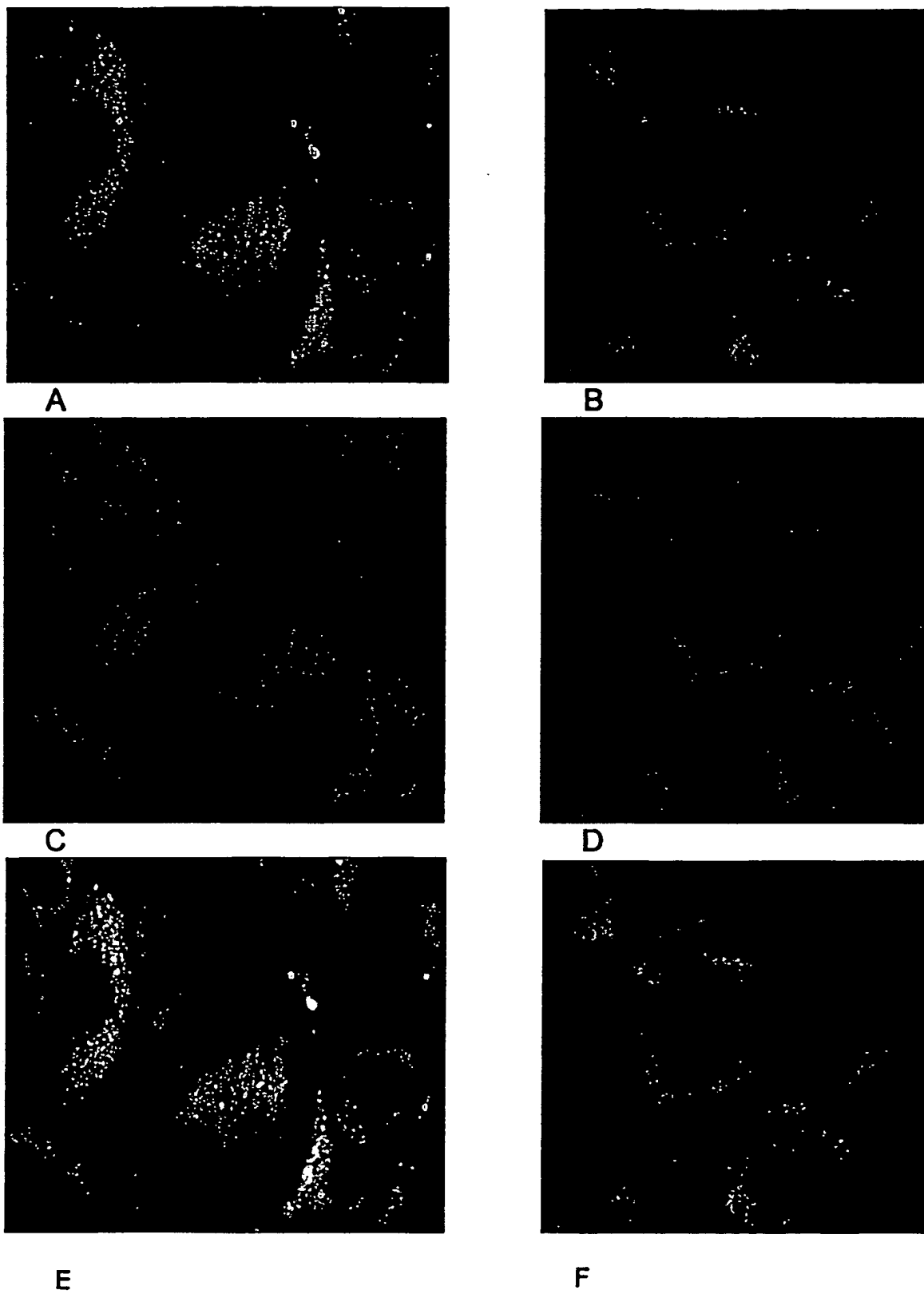


Fig. 9

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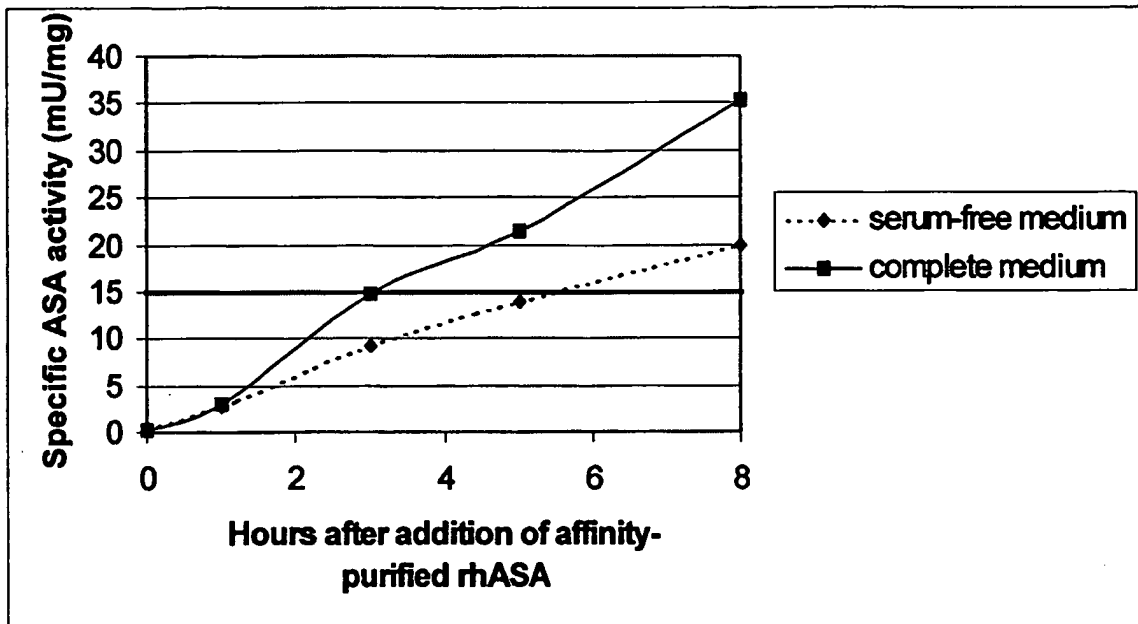


Fig. 10

11/16

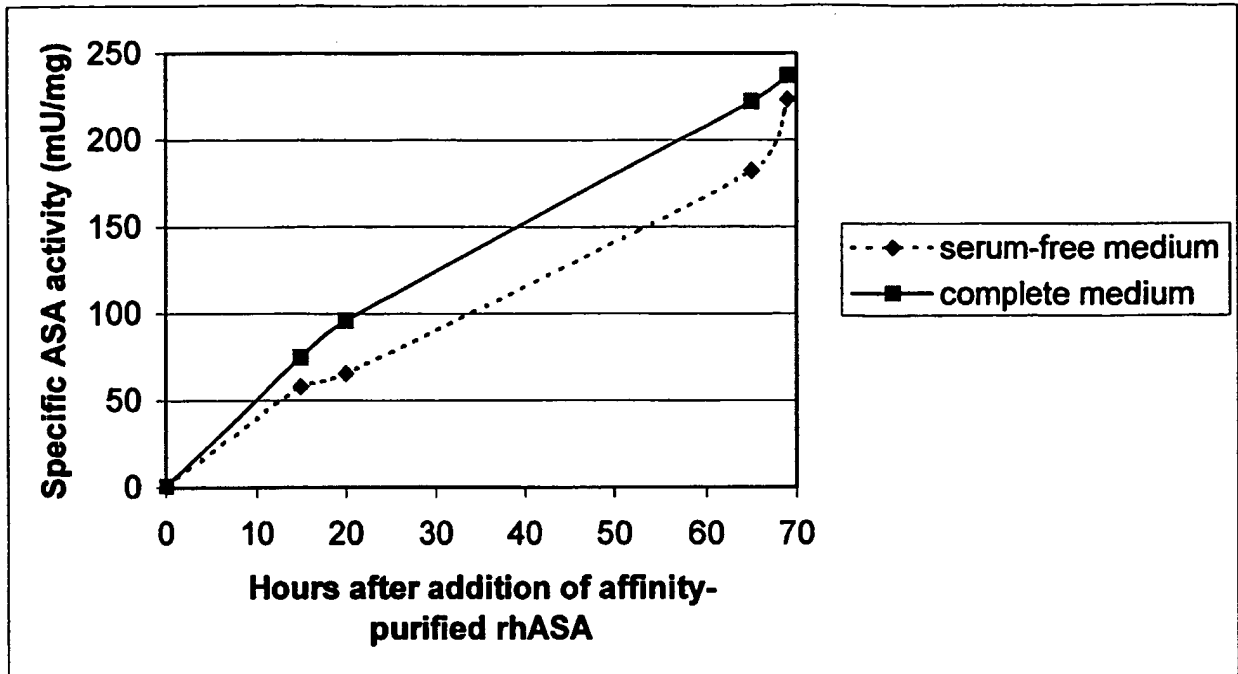


Fig. 11

12/16

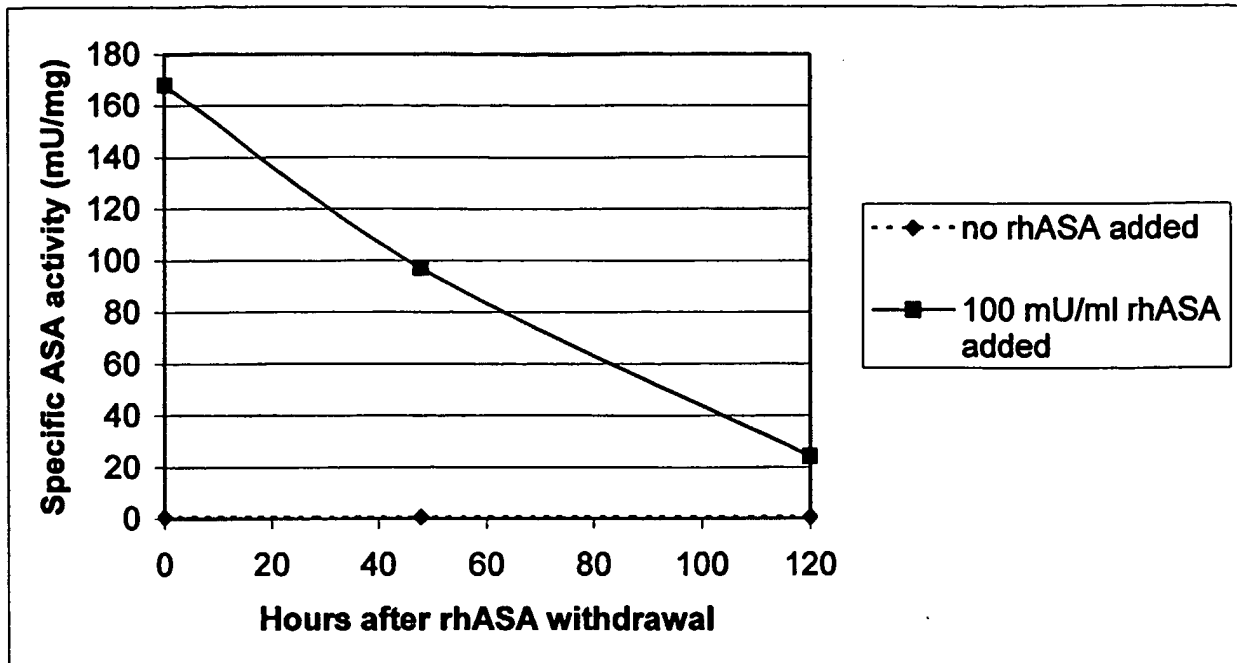


Fig. 12

13/16

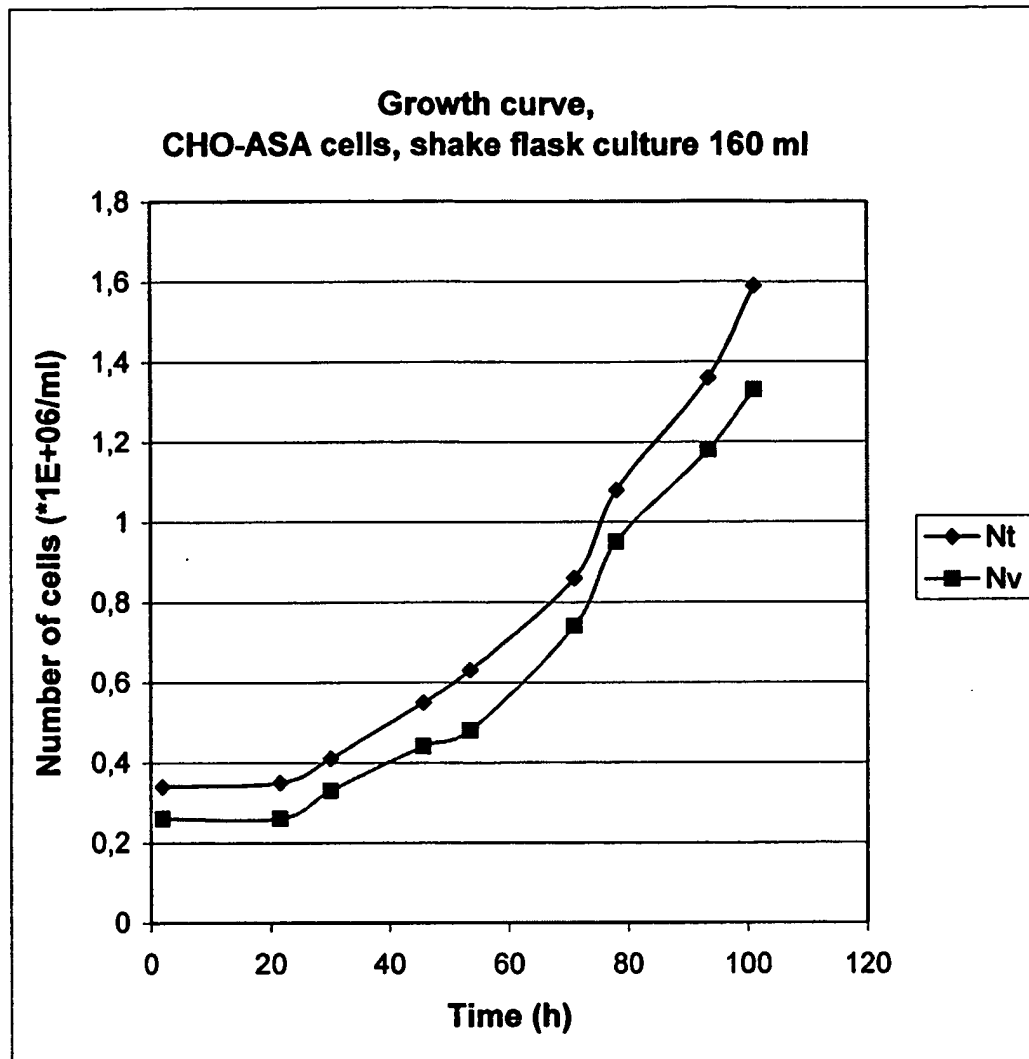
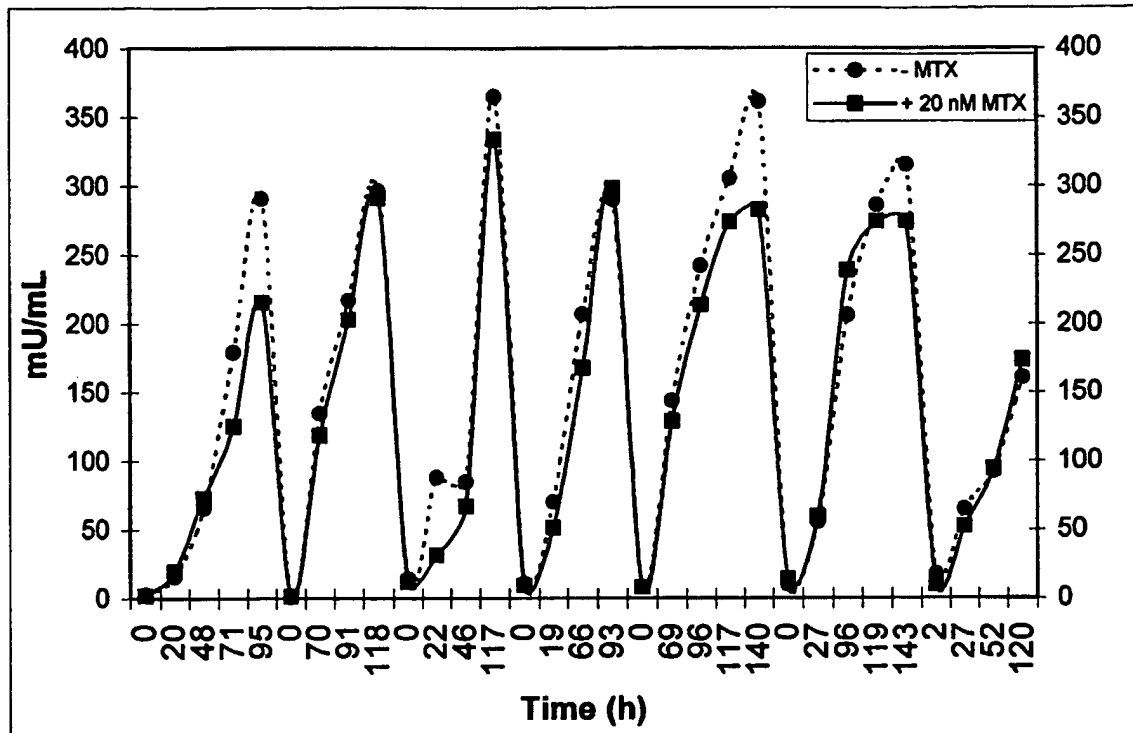


Fig. 13

14/16



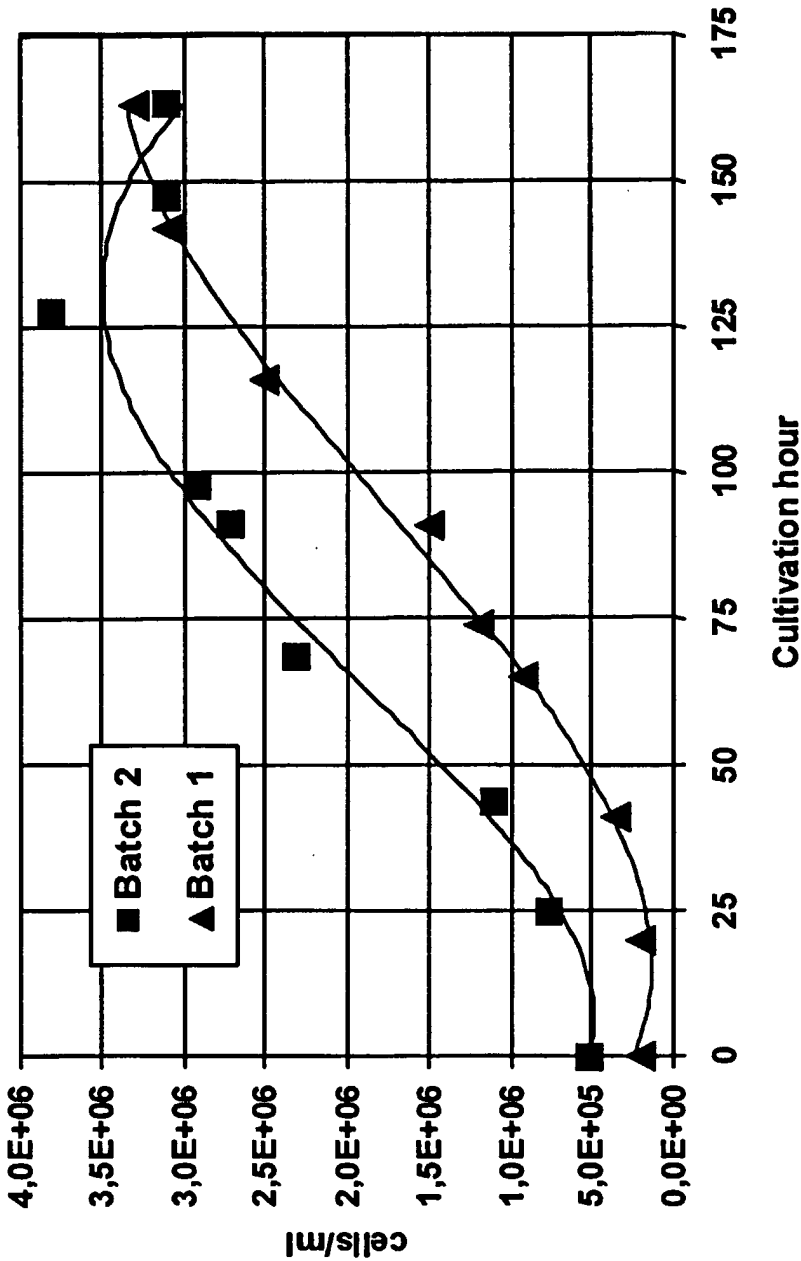


Fig. 15

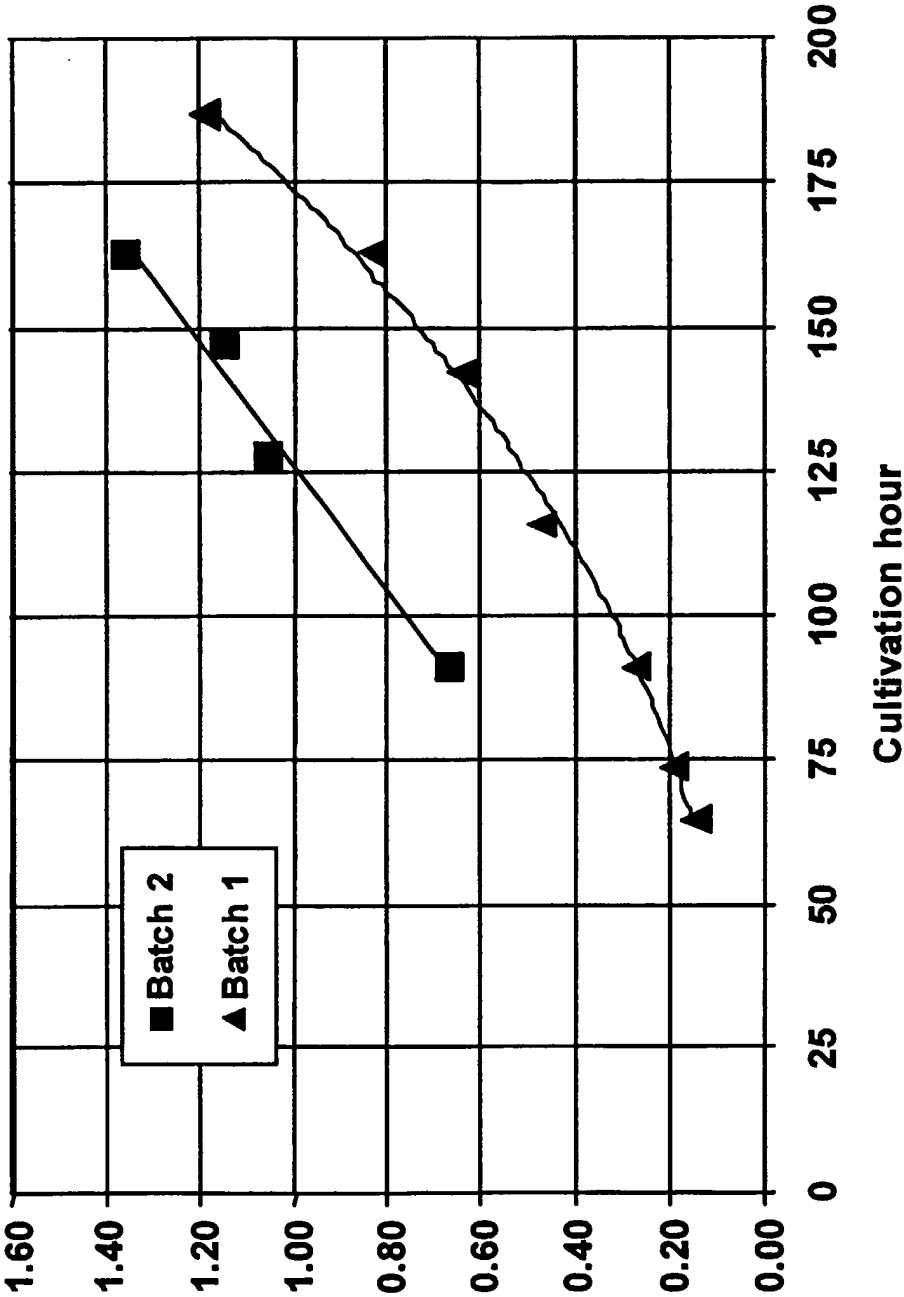


Fig. 16

SEQUENCE LISTING

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 Meher Irani
 Claes Andersson
 Cecilia Weigelt

<120> Production of recombinant human
 Arylsulfatase A

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<223> Btr I recognition site

<221> misc_feature

<222> (729)...(734)

<223> Sac II recognition site

<221> CDS

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Val Ala Arg Pro Pro Asn Ile Val Leu Ile Phe Ala Asp Asp Leu Gly	
1 5 10	
tat ggg gac ctg ggc tgc tat ggg cac ccc agc tct acc act ccc aac	144
Tyr Gly Asp Leu Gly Cys Tyr Gly His Pro Ser Ser Thr Thr Pro Asn	
15 20 25 30	
ctg gac cag ctg gcg gcg gga ggg ctg cgg ttc aca gac ttc tac gtg	192
Leu Asp Gln Leu Ala Ala Gly Gly Leu Arg Phe Thr Asp Phe Tyr Val	
35 40 45	
cct gtg tct ctg tgc aca ccc tct agg gcc gcc ctc ctg acc ggc cgg	240
Pro Val Ser Leu Cys Thr Pro Ser Arg Ala Ala Leu Leu Thr Gly Arg	

50	55	60	
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cgg ggg ggc ctg ccc ctg gag gag gtg acc gtg gcc gaa gtc ctg gct Arg Gly Gly Leu Pro Leu Glu Glu Val Thr Val Ala Glu Val Leu Ala 80 85 90			336
gcc cga ggc tac ctc aca gga atg gcc ggc aag tgg cac ctt ggg gtg Ala Arg Gly Tyr Leu Thr Gly Met Ala Gly Lys Trp His Leu Gly Val 95 100 105 110			384
ggg cct gag ggg gcc ttc ctg ccc ccc cat cag ggc ttc cat cga ttt Gly Pro Glu Gly Ala Phe Leu Pro Pro His Gln Gly Phe His Arg Phe 115 120 125			432
cta ggc atc ccg tac tcc cac gac cag ggc ccc tgc cag aac ctg acc Leu Gly Ile Pro Tyr Ser His Asp Gln Gly Pro Cys Gln Asn Leu Thr 130 135 140			480
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atg tcc cga ggc ggc tgc tcc ggt ctc ttg cgg tgt gga aag gga acg Met Ser Arg Gly Gly Cys Ser Gly Leu Leu Arg Cys Gly Lys Gly Thr 275 280 285			912
acc tac gag ggc ggt gtc cga gag cct gcc ttg gcc ttc tgg cca ggt			960

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His	Ile	Ala	Pro	Gly	Val	Thr	His	Glu	Leu	Ala	Ser	Ser	Leu	Asp	Leu		
		305					310					315					
ctg	cct	acc	ctg	gca	gcc	ctg	gct	ggg	gcc	cca	ctg	ccc	aat	gtc	acc	1056	
Leu	Pro	Thr	Leu	Ala	Ala	Leu	Ala	Gly	Ala	Pro	Leu	Pro	Asn	Val	Thr		
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Gln	Gly	Ser	Ala	His	Ser	Asp	Thr	Thr	Ala	Asp	Pro	Ala	Cys	His	Ala		
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Lys	Asp	Pro	Gly	Glu	Asn	Tyr	Asn	Leu	Leu	Gly	Gly	Val	Ala	Gly	Ala		
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Thr	Pro	Glu	Val	Leu	Gln	Ala	Leu	Lys	Gln	Leu	Gln	Leu	Leu	Lys	Ala		
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cag	tta	gac	gca	gct	gtg	acc	ttc	ggc	ccc	agc	cag	gtg	gcc	cgg	ggc	1440	
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cca	gct	tgc	tgc	cat	tgc	cca	gat	ccc	cat	gcc	tga					1524	
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pAsaExpl plasmid

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Met Gly Ala Pro Arg Ser Leu

1

5

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ctc ctg gcc ctg gct gct ggc ctg gcc gtt gca cgt ccg ccc aac atc 1182
Leu Leu Ala Leu Ala Ala Gly Leu Ala Val Ala Arg Pro Pro Asn Ile
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Val Leu Ile Phe Ala Asp Asp Leu Gly Tyr Gly Asp Leu Gly Cys Tyr
      25                30                35

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ggg cac ccc agc tct acc act ccc aac ctg gac cag ctg gcg gcg gga 1278
Gly His Pro Ser Ser Thr Thr Pro Asn Leu Asp Gln Leu Ala Ala Gly
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ggg ctg cgg ttc aca gac ttc tac gtg cct gtg tct ctg tgc aca ccc 1326
Gly Leu Arg Phe Thr Asp Phe Tyr Val Pro Val Ser Leu Cys Thr Pro
      60                65                70

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tct agg gcc gcc ctc ctg acc ggc cgg ctc ccg gtt cgg atg ggc atg 1374
Ser Arg Ala Ala Leu Leu Thr Gly Arg Leu Pro Val Arg Met Gly Met
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tac cct ggc gtc ctg gtg ccc agc tcc cgg ggg ggc ctg ccc ctg gag 1422
Tyr Pro Gly Val Leu Val Pro Ser Ser Arg Gly Gly Leu Pro Leu Glu
      90                95                100

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gag gtg acc gtg gcc gaa gtc ctg gct gcc cga ggc tac ctc aca gga 1470
Glu Val Thr Val Ala Glu Val Leu Ala Ala Arg Gly Tyr Leu Thr Gly
      105                110                115

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atg gcc ggc aag tgg cac ctt ggg gtg ggg cct gag ggg gcc ttc ctg 1518
Met Ala Gly Lys Trp His Leu Gly Val Gly Pro Glu Gly Ala Phe Leu

```

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gac cag ggc ccc tgc cag aac ctg acc tgc ttc ccg ccg gcc act cct Asp Gln Gly Pro Cys Gln Asn Leu Thr Cys Phe Pro Pro Ala Thr Pro	155	160	165	1614
tgc gac ggt ggc tgt gac cag ggc ctg gtc ccc atc cca ctg ttg gcc Cys Asp Gly Gly Cys Asp Gln Gly Leu Val Pro Ile Pro Leu Leu Ala	170	175	180	1662
aac ctg tcc gtg gag gcg cag ccc ccc tgg ctg ccc gga cta gag gcc Asn Leu Ser Val Glu Ala Gln Pro Pro Trp Leu Pro Gly Leu Glu Ala	185	190	195	1710
cgc tac atg gct ttc gcc cat gac ctc atg gcc gac gcc cag cgc cag Arg Tyr Met Ala Phe Ala His Asp Leu Met Ala Asp Ala Gln Arg Gln	200	205	210	1758
gat cgc ccc ttc ttc ctg tac tat gcc tct cac cac acc cac tac cct Asp Arg Pro Phe Phe Leu Tyr Tyr Ala Ser His His Thr His Tyr Pro	220	225	230	1806
cag ttc agt ggg cag agc ttt gca gag cgt tca ggc cgc ggg cca ttt Gln Phe Ser Gly Gln Ser Phe Ala Glu Arg Ser Gly Arg Gly Pro Phe	235	240	245	1854
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ggt ctc ttg cgg tgt gga aag gga acg acc tac gag ggc ggt gtc cga Gly Leu Leu Arg Cys Gly Lys Gly Thr Thr Tyr Glu Gly Gly Val Arg	300	305	310	2046
gag cct gcc ttg gcc ttc tgg cca ggt cat atc gct ccc ggc gtg acc Glu Pro Ala Leu Ala Phe Trp Pro Gly His Ile Ala Pro Gly Val Thr	315	320	325	2094
cac gag ctg gcc agc tcc ctg gac ctg ctg cct acc ctg gca gcc ctg His Glu Leu Ala Ser Ser Leu Asp Leu Leu Pro Thr Leu Ala Ala Leu	330	335	340	2142
gct ggg gcc cca ctg ccc aat gtc acc ttg gat ggc ttt gac ctc agc Ala Gly Ala Pro Leu Pro Asn Val Thr Leu Asp Gly Phe Asp Leu Ser	345	350	355	2190
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Pro Leu Leu Leu Gly Thr Gly Lys Ser Pro Arg Gln Ser Leu Phe Phe
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 Gly Lys Tyr Lys Ala His Phe Phe Thr Gln Gly Ser Ala His Ser Asp
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 Phe Gly Pro Ser Gln Val Ala Arg Gly Glu Asp Pro Ala Leu Gln Ile
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 Asp Pro His Ala *
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6591

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cg 62